ผลของแอลคาร์นิทีนต่อการเจริญเป็นไข่สุก ความทนทานต่อการแช่แข็งและการพัฒนาเป็นตัวอ่อนของไข่โคหลังการปฏิสนธิ

นางสาวธีวรา พงษ์นิมิตร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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EFFECTS OF L-CARNITINE ON MATURATION, CRYO-TOLERANCE AND EMBRYO DEVELOPMENTAL COMPETENCE OF BOVINE OOCYTES AFTER *IN VITRO* FERTILIZATION

Teewara Phongnimitr

A Thesis Submitted in Partial Fulfillment of the Requirements for the

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EFFECTS OF L-CARNITINE ON MATURATION, CRYO-TOLERANCE AND EMBRYO DEVELOPMENTAL COMPETENCE OF BOVINE OOCYTES AFTER *IN VITRO* FERTILIZATION

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

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ธีวรา พงษ์นิมิตร : ผลของแอลคาร์นิทีนต่อการเจริญเป็นไข่สุก ความทนทานต่อการแช่แข็ง และการพัฒนาเป็นตัวอ่อนของไข่โคหลังการปฏิสนธิ (EFFECTS OF L-CARNITINE ON MATURATION, CRYO-TOLERANCE AND EMBRYO DEVELOPMENTAL COMPETENCE OF BOVINE OOCYTES AFTER *IN VITRO* FERTILIZATION) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.รังสรรค์ พาลพ่าย, 71 หน้า.

การเลี้ยงไข่อ่อนให้สุกภายในห้องปฏิบัติการเป็นขั้นตอนหนึ่งที่สำคัญในการกำหนดถึงความสำเร็จของการผลิตตัวอ่อนภายในห้องปฏิบัติการ วัตถุประสงค์ของงานวิจัยคือ ศึกษาผลของแอลคาร์นิทีนที่เติมในน้ำยาสำหรับเลี้ยงไข่อ่อนต่อการเจริญเป็นไข่สุกและการพัฒนาเป็นตัวอ่อนของไข่โค รวมทั้งการรอดชีวิตและการเจริญเป็นตัวอ่อนระยะบลาสโตซิสของไข่ที่แช่แข็งแบบ vitrification นอกจากนี้ยังศึกษาผลของแอลคาร์นิทีนที่เติมในขั้นตอนการปฏิสนธิและการเลี้ยงตัวอ่อนต่อการเจริญเป็นตัวอ่อนและคุณภาพของตัวอ่อนด้วย ในการทดลองที่ 1 และ 2 ไข่อ่อนโคจะถูกเลี้ยงในน้ำยาสำหรับเลี้ยงไข่อ่อนที่มีความเข้มข้นของแอลคาร์นิทีน 0.3, 0.6 และ 1.2 mg/mL กลุ่มที่ไม่เติมแอลคาร์นิทีนเป็นกลุ่มควบคุม จากนั้นไข่จะถูกตรึงและย้อมด้วยสี aceto-orcein เพื่อตรวจดูการเจริญเป็นไข่สุก จากผลการทดลองพบว่าแอลคาร์นิทีนที่ความเข้มข้น 0.3 และ 0.6 mg/mL มีอัตราการเจริญเป็นไข่สุกสูงกว่ากลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ ในขณะที่กลุ่ม 1.2 mg/mL อัตราการเจริญเป็นไข่สุกไม่มีความแตกต่างอย่างมีนัยสำคัญกับกลุ่มควบคุม ต่อมาเมื่อนำไข่ที่เลี้ยงในห้องปฏิบัติการ ทำการปฏิสนธิกับอสุจิในหลอดแก้วแล้วนำมาเลี้ยงต่อในน้ำยาสำหรับเลี้ยงตัวอ่อน พบว่าอัตราการเจริญเป็นตัวอ่อนระยะบลาสโตซิสในกลุ่ม 0.6 mg/mL เพิ่มขึ้นอย่างมีนัยสำคัญ แต่กลุ่ม 1.2 mg/mL อัตราการเจริญสู่ระยะบลาสโตซิสลดลงอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับกลุ่มควบคุม เมื่อนับจำนวนเซลล์ของตัวอ่อนระยะบลาสโตซิสพบว่าจำนวนมวลเซลล์ชั้นใน (ICM cells) และเซลล์โทรเฟคโตเดอร์ม (TE cells) ไม่มีความแตกต่างกันทั้งกลุ่มที่เติมและไม่เติมแอลคาร์นิทีน

ในการทดลองที่ 3 ทำการศึกษาผลของแอลคาร์นิทีนต่อการแช่แข็งไข่โค โดยไข่จากกลุ่มที่มีความเข้มข้นของแอลคาร์นิทีน 0.6 mg/mL จะถูกนำมาแช่แข็ง จากนั้นไข่จะถูกละลายในสารละลายที่เจือจางเป็นลำดับขั้นและทำการปฏิสนธิกับอสุจิในหลอดแก้ว จากผลการทดลองพบว่าอัตราการรอดชีวิตหลังการละลาย อัตราการแบ่งตัว และอัตราการเจริญเป็นตัวอ่อนระยะบลาสโตซิสไม่มีความแตกต่างกันในกลุ่มที่แช่แข็ง และไม่พบความแตกต่างของจำนวนมวลเซลล์ชั้นในและเซลล์โทเฟคโตเดอร์มของตัวอ่อนระยะบลาสโตซิสที่ได้จากไข่แช่แข็ง นอกจากนี้ตัวอ่อนระยะ บลาสโตซิสจากไข่ที่แช่แข็ง ยังพบว่ามีจำนวนมวลเซลล์ชั้นในและเซลล์โทเฟคโตเดอร์มต่ำกว่ากลุ่มไข่สดที่ไม่ได้แช่แข็งทั้งที่เติมและไม่เติมแอลคาร์นิทีนอีกด้วย

ในการทดลองที่ 4 ความเข้มข้นของแอลคาร์นิทีนที่ 0.6 mg/mL ยังนำมาการศึกษาต่อเนื่องถึงผลของแอลคาร์นิทีนที่เติมในน้ำยาสำหรับการปฏิสนธิและน้ำยาสำหรับเลี้ยงตัวอ่อนต่อการเจริญเป็นตัวอ่อนโค จากผลการทดลองพบว่าการเติมแอลคาร์นิทีนในน้ำยาสำหรับการปฏิสนธิและน้ำยาสำหรับเลี้ยงตัวอ่อนไม่มีผลต่อการเจริญเป็นตัวอ่อนระยะบลาสโตซิส

การทดลองนี้สรุปได้ว่าการเติมแอลคาร์นิทีนในน้ำยาสำหรับเลี้ยงไข่อ่อนโค สามารถเพิ่มอัตราการเจริญเป็นไข่สุกและอัตราการเจริญเป็นตัวอ่อนโคหลังการปฏิสนธิได้ แต่แอลคาร์นิทีนไม่มีผลต่อไข่โคเมื่อไข่โคถูกแช่แข็งแบบ vitrification นอกจากนี้การเติมแอลคาร์นิทีนตลอดกระบวนการเลี้ยงตัวอ่อน (น้ำยาสำหรับการปฏิสนธิและน้ำยาสำหรับเลี้ยงตัวอ่อน) ไม่มีผลต่อการพัฒนาเป็นตัวอ่อนและคุณภาพของตัวอ่อนโค

มหาวิทยาลัยเทคโนโลยีสุรนารี

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2555 ลายมือชื่ออาจารย์ที่ปรึกษา_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม _____

TEEWARA PHONGNIMITR : EFFECTS OF L-CARNITINE ON MATURATION, CRYO-TOLERANCE AND EMBRYO DEVELOPMENTAL COMPETENCE OF BOVINE OOCYTES AFTER *IN VITRO* FERTILIZATION. THESIS ADVISOR : ASSOC. PROF. RANGSUN PARNPAI, Ph.D., 71 PP.

BOVINE/*IN VITRO* MATURATION/L-CARNITINE/VITRIFICATION/ OOCYTE/EMBRYO DEVELOPMENT

In vitro maturation (IVM) is a crucial step in determining the success of *in vitro* embryo production (IVP). The aim of this study was to evaluate the effects of L-carnitine addition to IVM medium on nuclear maturation and embryo development of bovine oocytes, including survival and blastocyst rate of vitrified-oocytes. Moreover, the effects of L-carnitine during *in vitro* fertilization (IVF) and *in vitro* culture (IVC) on the developmental potential and quality of embryos were also examined. In Experiments 1 and 2, immature bovine oocytes were matured in IVM medium supplemented with 0.3, 0.6 and 1.2 mg/mL of L-carnitine (0.3, 0.6 and 1.2 groups, respectively). Oocytes matured in 0 mg/mL of L-carnitine were treated as a control group. The nuclear maturation of oocytes was assessed by fixing and staining with aceto-orcein dye. A significantly higher maturation rate of oocytes was obtained for 0.3 and 0.6 mg/mL groups compared with the control ($P< 0.05$). Although the maturation rate in the 1.2 group (75.8%) appeared higher, it was not significantly different when compared with the control group (67.8%). After IVM, the oocytes were subjected to IVF and cultured *in vitro.* The blastocyst formation rate in the 0.6

mg/mL group was significantly improved, whereas the rate in the 1.2 mg/mL group was significantly decreased when compared with the control group ($P< 0.05$). However, there was no significant difference in TE and ICM cell numbers among those groups.

In Experiment 3, 0.6 mg/mL of L-carnitine was used to investigate the effects of L-carnitine in IVM of bovine oocytes on their cryopreservation. The survival, cleavage and blastocyst rates were not significantly different between vitrified groups, and additionally, the numbers of TE and ICM cells of blastocysts in vitrified groups were not significantly different and were lower than those of fresh groups irrespective of L-carnitine treatment.

In Experiment 4, 0.6 mg/mL of L-carnitine was used to investigate the effects of L-carnitine in IVF and IVC media on embryo developmental competence. Supplementation of IVF and IVC media with L-carnitine had no positive effect on embryo development to the blastocyst stage.

In conclusion, the supplementation of L-carnitine during IVM of bovine oocytes improved their nuclear maturation and subsequent embryo development after IVF, but when they were vitrified the improving effects were neutralized. Additionally, L-carnitine supplementation throughout the entire IVP process (IVF and IVC) did not improve developmental capacity and quality of resulting bovine embryos.

School of Biotechnology
Academic Year 2012

Student's Signature _____
Advisor's Signature _____
Co-advisor's Signature _____

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Teewara Phongnimitr

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LIST OF ABBREVIATIONS

AI	=	artificial insemination
GV	=	germinal vesicle
GVBD	=	germinal vesicle breakdown
ICM	=	inner cell mass
IVC	=	*in vitro* culture
IVF	=	*in vitro* fertilization
IVM	=	*in vitro* maturation
IVP	=	*in vitro* production
µg	=	microgram
µL	=	microliter
mg	=	miligram
mil	=	million
min	=	minute
mL	=	mililiter
OPS	=	open-pulled straws
PBS	=	phosphate buffered saline
SCNT	=	somatic cell nuclear transfer
SSV	=	solid surface vitrification
TCM	=	tissue culture medium
TE	=	trophectoderm cells

มหาวิทยาลัยเทคโนโลยีสุรนารี

CHAPTER I

INTRODUCTION

1.1 Background

Bovine species has long been a focus on research due to its importance in agriculture (Hodges and Stice, 2003) of many countries in terms of milk production, meat and labor. Interestingly, bovine has used as a model to study the human. Bovine and human share other characteristic such as endocrine pathways, paracrine and autocrine pathways, the size of ovaries and oocytes, embryonic genome activation, the development of one pre-ovulatory follicle during each cycle and interaction with culture medium (Chian et al., 2004). Although the agriculturists can produce new calves over the years, the outcome is still low. The remained problems and challenges, possibly due to their poor reproductive systems. Bovine has several intrinsic factors that influence their reproductive potential such as age, physiological status and genetic differences (Kafi and McGowan, 1997; Orihuela, 2000). Moreover, the extrinsic factors including season, nutrition, location and management (Calder and Rajamahendran, 1992; Kafi and McGowan, 1997; Orihuela, 2000; Sakhong et al., 2011) could also impair their fertile ability. To increase the economic benefit of bovine, in *vitro* embryo production (IVP) including *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* embryo culture (IVC) offer the potential for enhancing quality and yield of embryo, resulting in high pregnancy rate and subsequent high live offspring production.

IVM is the process that involves the meiotic maturation of oocyte from germinal vesicle (GV) stage to metaphase-II (MII) stage. IVM is suggested to be the first and crucial step determining the success of *in vitro* embryo production, because the number and quality of MII oocytes after IVM directly influence embryo development rate. Variations in maternal mRNA synthesis or accumulation during oocyte maturation may affect the *in vitro* development of embryo until zygotic gene activation (Mouatassim et al., 1999). Abdoon et al. (2001) found that good quality oocytes had significantly higher cleavage, and developmental rates up to morula stage compared with oocytes of fair or poor quality. Previous studies indicated that IVM can result in deleterious effects on the organization of the spindle fiber and chromosomes of immature oocytes (Li et al., 2006). This may cause a high incidence of chromosomal abnormality in embryos derived from IVM protocol (Requena et al., 2009), which possibly lead to low blastocyst formation, implantation and pregnancy rates. One of the major factors affecting efficiency of IVM is culture condition. It is believed that culture condition is the most important factor, because different culture conditions could affect rate of oocyte maturation and proper embryonic development (Jee et al., 2008). Nevertheless, oocytes culture under high oxygen tension *in vitro* (20% O_2) can produce high number of free radicals species, such as reactive oxygen species (ROS). It is known that ROS can bind to cellular macromolecule and can promote lipid peroxidation which resulting in cell damage (Comporti, 1989). To solve these problems, many scientists have tried to improve the IVM medium conditions of bovine by adding gonadotrophin hormones such as luteinizing hormone (LH) (Zuelke and Brackett, 1990) and/or follicle stimulating hormone (FSH) (Anderiesz et al., 2000), serum from different sources such as fetal calf serum (FCS) or oestrus calf

serum (ECS) (Fukui and Ono, 1989) and bovine follicular fluid (bFF) (Ali et al., 2004). In addition, growth factors such as epidermal growth factor (EGF) added to bovine IVM medium, can increase the proportion of oocytes undergo to blastocyst stage (Harper and Brackett, 1993; Oyamada and Fukui, 2004). To protect cells from oxidative stress, various antioxidants including cysteamine (de Matos et al., 2002; Balasubramanian and Rho, 2007), cysteine, *N*-acetyl-L-cystein, catalase and superoxide dismutase (Ali et al., 2003) were also used. Therefore, exploring the factors which essential for bovine oocyte metabolism is a critical step to optimize IVP outcome.

Interestingly, mitochondrial organization and ATP levels of oocytes can affect their developmental capacity after IVF (Stojkovic et al., 2001). Previous study reported that L-carnitine which plays a crucial role in β-oxidation by transporting the fatty acid into mitochondria for generate the ATP, can enhance oocyte maturation and embryonic development in porcine (Wu et al., 2011; Somfai et al., 2011). Moreover, L-carnitine cloud improve blastocyst development rate in mouse embryos (Abdelrazik et al., 2009). When derivatives of L-carnitine were added to culture medium, the rate of bovine embryos developed to blastocysts was increased (Ono et al., 2007). Other important function of L-carnitine, it acted as an antioxidant by reducing the levels of ROS, H_2O_2 and increasing glutathione concentration during IVM of porcine oocyte (Wu et al., 2011; Somfai et al., 2011). In addition, L-carnitine can increase the levels of vitamin C, vitamin E (Rani and Panneerselvam, 2001), superoxide dismutase (Cetinkaya et al., 2006) and catalase (Izgut-Uysal et al., 2001) which play an important role in defense mechanism to protect cells from oxidative stress. And also, L-carnitine is a beneficial effect in protecting cells from apoptosis via reducing DNA

fragmentation (Qi et al., 2006), inhibiting mitochondrial dysfunction and mitochondrial DNA injury (Chang et al., 2002), and stabilizing mitochondrial membranes (Pillich et al., 2005). Besides, the high lipid content in oocyte cytoplasm is one of the major causes in poor embryonic development of bovine oocytes following vitrification, because high amount of lipid droplets make the oocyte more sensitive to chilling injury (Boonkusol et al., 2007). Recently, Somfai et al. (2011) found that the density of lipid droplets decreased when porcine oocytes were cultured in IVM medium supplementation with L-carnitine. Thus pre-treated bovine oocytes with L-carnitine following vitrification to reduce lipid droplets that lead to reduce in cryoinjury may be provide the new technique for improvement the oocyte cryopreservation outcomes.

Up to now, the potential roles of L-carnitine on cryopreservation of matured bovine oocytes have not yet been examined. In addition, there has not yet been reported about the effect of L-carnitine addition to IVM medium of bovine oocytes on nuclear maturation and their developmental competence. Hence, the aims of this study were to determine the effects of L-carnitine added to IVM medium on 1) oocyte nuclear maturation and 2) embryo developmental competence after IVF, including 3) survival and blastocyst rate of vitrified-bovine oocytes after warming. In addition, 4) the beneficial effects of L-carnitine added to IVF and IVC medium on embryo developmental competence were also assessed.

1.2 Research objectives

1.2.1 To evaluate the effects of L-carnitine supplemented to IVM medium on nuclear maturation of bovine oocytes.

1.2.2 To evaluate the effects of L-carnitine supplemented to IVM medium on cleavage and blastocyst rates of bovine oocytes after IVF.

1.2.3 To investigate the survival rate and embryo developmental rate of vitrified-warmed bovine oocytes matured in IVM medium supplemented with L-carnitine and fertilized by IVF.

1.2.4 To determine the effects of L-carnitine when added to IVF and IVC medium on embryo developmental rate of bovine oocytes.

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CHAPTER II

LITERATURE REVIEW

2.1 Introduction of Bovine

Bovine belongs to the genus *Bos* within family Bovidae (Dobson and Kamonpatana, 1986). They have many distinct species such as, *Bos indicus* (cattle of Asia and Africa) and *Bos taurus* (cattle of Europe) (Dobson and Kamonpatana, 1986). In Thailand, *Bos indicus* are the Thai native bovine, which are often used as draught animals especially in rice fields (Jaturasitha et al., 2009). *Bos indicus* are the bovine in tropical climate. They can adapt to high temperature, tolerate harsh sunshine, and are resistant to ticks and disease (Sakhong et al., 2011). But they are poorer in reproductive capability than *Bos Taurus* (temperate bovine) such as longer lengths of gestation and postpartum anestrus, puberty at older age, shorter duration of estrus and more often express estrus during the night (Baruselli et al., 2006). The low reproductive efficiency in bovine is a major problem limiting the genetic improvement in this species. There were many studies reported that their reproductive efficiency are related with age, physiology, management, nutrition and climate (Ryan et al., 1992; Kafi and McGowan, 1997; Orihuela, 2000; Sakhong et al., 2011).

Although superovulation following AI results in production of more transferable embryos, the procedure may also induce abnormalities in follicular development, oocyte maturation, and ovulation (Kafi and McGowan, 1997). Then, the in vitro embryo production became an interesting method for improving the bovine

outcome because it can produce about 3.4 times more embryos and 3.2 times more pregnancies in one superovulation per donor (Mapletoft and Hasler, 2005). IVP consist of IVM, IVF and IVC. IVM is suggested to be a first and crucial step determining the success in *in vitro* embryo production. Therefore, exploring the factors that essential for bovine oocyte metabolism is a critical step to optimize IVP outcome.

2.2 *In vitro* maturation in bovine

Since the normal calves were born from oocyte matured in vitro (Fukuda et al., 1990), IVM became an interesting option to avoid the problems derived from superovulation. IVM does not require the treatment of gonadotrophins for stimulation and time for estrus observation. IVM refers to the maturation of oocyte in vitro, which comprises of nuclear and cytoplasmic maturation. Nuclear maturation is the resumption of meiosis and progression from germinal vesicle breakdown (GVBD) to metaphase-II (MII) stage and cytoplasmic maturation is the preparation of oocyte cytoplasm for fertilization and embryonic development (Lin and Hwang, 2006). It is known that several mRNA and proteins which essential for further development of embryo are synthesized and accumulated during oocyte maturation. Poor quality of oocyte is a major restriction in IVF outcomes which affects the in vitro development of embryo to blastocyst stage. Abdoon et al. (2001) confirmed that the embryos from good quality oocyte could develop to morula stage whereas all embryos from oocyte of poor quality arrested at two to sixteen cells stage. There are many factors which can affect efficacy of oocyte in vitro such as type of media, media compositions, pH, time, and / or temperature. To induce full maturation of bovine oocytes, types of hormones,

different source of proteins and other important factors, such as antioxidants or growth factors were added to IVM medium. FSH and LH have an important role in the regulation of follicular growth and oocyte meiotic maturation (Anderiesz et al., 2000). When LH was added to serum-free IVM medium, the cleavage and blastocyst rate significantly improved (Zuelke and Brackett, 1990). Although the combination of FSH and LH during IVM did not affect the number of inner cell mass (ICM) or trophectoderm (TE) cells, but the maturation of immature oocytes to MII stage and the proportion of embryo developed to blastocyst was significantly increased (Anderiesz et al., 2000). Jee et al. (2008) indicated that protein may serve as a nitrogen source and act as chelator of toxic metal ions within culture media. The positive effects of fetal calf serum and estrus cow serum on bovine oocyte maturation, fertilization and embryo development *in vitro* has also been reported (Fukui and Ono, 1989). Ali et al. (2004) has proved that the bovine follicular fluid (bFF) originating from competent follicle (>8 mm) increased the developmental competence of abattoir-derived oocyte, compared with bFF from small follicle (2-5 mm). Furthermore, the growth factors such as epidermal growth factors (EGF) act as both autocrine and paracrine can improve oocyte quality resulting in higher blastocyst rate (Harper and Brackett, 1993; Oyamada and Fukui, 2004).

Nevertheless, oocytes cultured under high oxygen tension *in vitro* (20% O_2) can produce high number of free radicals species, such as reactive oxygen species (ROS). Oxygen is one type of ROS, which can diffuse and pass through cell membranes and alter most types of cellular molecules such as lipids, proteins and nucleic acids (Roushandeh and Roudkenar, 2009). It is known that ROS can induce mitochondrial dysfunction, DNA, RNA and protein damage as well as inhibiting

sperm-oocyte fusion (Ali et al., 2003). In mammals, glutathione (GSH) plays an important role to protect cells from oxidative stress. Low molecular weight thiol compounds, such as cysteamine supplemented during IVM increase intracellular glutathione synthesis and improve cleavage and blastocyst rate in bovine species, although no positive effect on nuclear maturation (de Matos et al., 2002, Oyamada and Fukui, 2004 and Balasubramanian and Rho, 2007). Although, IVM has been studied in bovine over the years but the positive effects of L-carnitine during oocytes matured *in vitro*, embryo developmental competence and oocytes cryopreservation in bovine have not yet been examined.

2.3 L-carnitine

Carnitine (3-hydroxy-4-N-trimethylaminobutyric acid) (Figure 1) was first isolated from bovine muscle in 1905 and only the L-isomer was found bioactive (Zhou et al., 2007). Carnitine synthesis is a multiple-step process, which is synthesized from two essential amino acids lysine and methionine (Steiber et al., 2004). It is a small water-soluble molecule important for normal oxidation of fatty acid by mitochondria in mammalian fat metabolism (Vanella et al., 2000). L-carnitine transports the long-chain fatty acid from cytosol across the inner membrane of mitochondria to generate the ATP (Figure 2). This transport system consists of three enzyme proteins, 1) carnitine palmitoyltransferase I (CPT-I), 2) acylcarnitine:carnitinetranslocase (CACT) and 3) carnitine palmitoyltransferase II (CPT-II) (Steiber et al., 2004). L-carnitine is an essential cofactor of CPT-I, a key enzyme for of long chain fatty acid oxidation (Abdel-aleem et al., 1998).

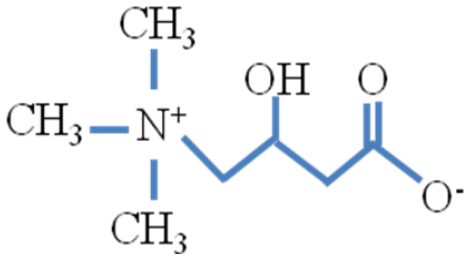

Figure 1. Carnitine configuration

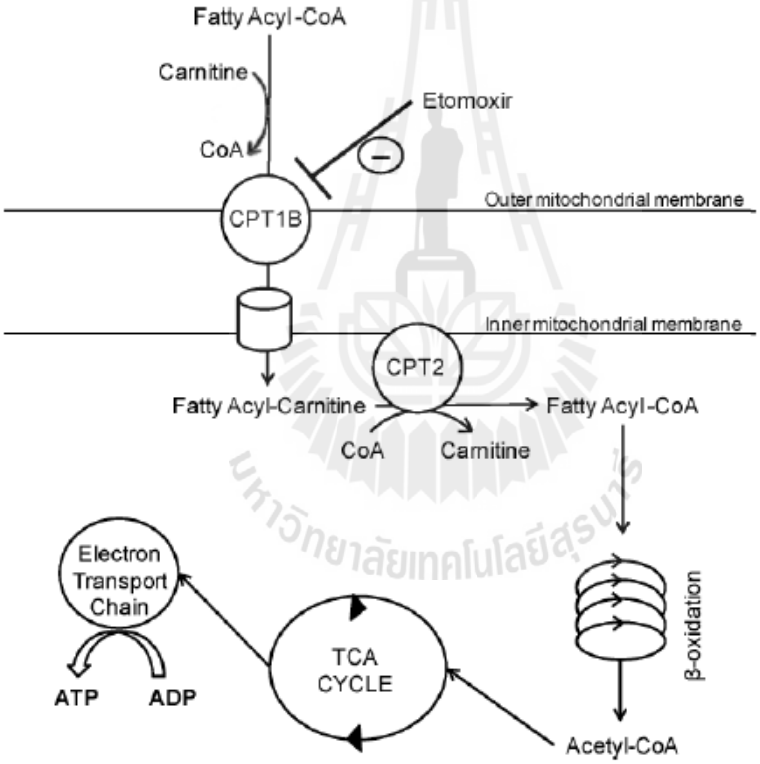

Figure 2. Schematic representation of β-oxidation pathway (Dunning et al., 2010)

Enhancement of lipid metabolism, mitochondrial function, nuclear maturation and cleavage ability of porcine oocytes by L-carnitine has been reported (Somfai et al., 2011). Above results showed that L-carnitine significantly increased the rates of

MII stage oocytes and cleavage of embryos. Moreover, the density of active mitochondria was significantly higher and the density of lipid droplets was significantly lower in L-carnitine-treated oocytes compared with control (Somfai et al., 2011). They concluded that, enhancing mitochondrial functions by L-carnitine improved oocyte maturation and cleavage underlining the importance of lipid metabolism for nuclear and cytoplasmic maturation of oocytes. In the same year (2011), Wu et al. also reported that the adding L-carnitine to IVM medium accelerated nuclear maturation of porcine oocyte and increased the percentage of blastocyst development after parthenogenetic activation. When L-carnitine or its derivatives were supplemented to culture medium, the cleavage rates in bovine and blastocyst rates in mouse embryos improved (Ono et al., 2007; Abdelrazik et al., 2009). Moreover, L-carnitine has an antioxidant activity by acts as a free radical scavenger in aging (Rani and Panneerselvam, 2001), reducing the levels of ROS and H_2O_2 and increasing glutathione concentration during IVM or IVC (Abdelrazik et al., 2009; Wu et al., 2011; Somfai et al., 2011). And also it can increase the levels of vitamin C, vitamin E (Rani and Panneerselvam, 2001), superoxide dismutase (Cetinkaya et al., 2006) and catalase (Izgut-Uysal et al., 2001) which play an important role in defense mechanism to protect cells from oxidative stress. In addition, L-carnitine is an effective anti-apoptotic agent by inhibiting the activity of caspase-3 which plays a crucial role in mediating apoptotic DNA fragmentation during apoptosis (Qi et al., 2006). Reduction of apoptosis through the mitochondrial pathway by supplementation of acetyl-L-carnitine to culture medium of mouse fibroblasts was demonstrated (Pillich et al., 2005). The results showed that acetyl-L-carnitine is able to stabilize mitochondrial membranes and to stimulate mitochondrial metabolism. Furthermore,

Chang et al. (2002) found that the mitochondrial dysfunction, mitochondrial DNA injury, lipid peroxidation and apoptosis of epithelial cells were strongly inhibited by L-carnitine. The other beneficial effects of L-carnitine are protection oocyte cytoskeleton damage and embryo apoptosis induced by incubation in peritoneal fluid from patients with endometriosis (Mansour et al., 2009). This strategy may help improvement in *vitro* fertilization outcome in these patients. Therefore, supplementation of L-carnitine during in *vitro* maturation of bovine oocytes may enhance the nuclear maturation and improve the embryo development to blastocyst stage. The effects of L-carnitine supplementation to IVM medium on maturation, fertilization and embryo development were summarized in Table 1.

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Table 1. The improvement of maturation, fertilization and embryo development after supplementation of L-carnitine in IVM medium

Experiments	L-carnitine Treatments	Improvement				References
		% Maturation	% Fertilization	% Cleavage	% Blastocyst	
Porcine	0 mg/mL	-	Not determined	-	-	Wu et al., 2011
	0.25 mg/mL	No		No	No	
	0.5 mg/mL	No		No	Yes	
	1 mg/mL	No		No	No	
	2 mg/mL	Decrease		No	Decrease	
Porcine	0 mg/mL	-	-	-	-	Somfai et al., 2011
	0.3 mg/mL	No	No	No	No	
	0.6 mg/mL	Yes	No	Yes	No	
	1.2 mg/mL	Yes	No	Yes	No	
	2.5 mg/mL	Yes	No	Yes	No	
	5 mg/mL	Yes	No	No	No	
	10 mg/mL	No	No	No	No	

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2.4 Cryopreservation of bovine oocytes

Cryopreservation is an important tool for preservation and management of mammalian gamete or embryos to be used in the future. It is useful for widespread application of assisted reproductive technology, such as preserving endangered species, and providing oocytes for IVF or somatic cell nuclear transfer (SCNT) (Yang et al., 2008). Despite, embryos have been successfully cryopreserved in many species but oocytes cryopreservation still remains a problem and interesting question. Because oocytes are more sensitive to suboptimal conditions during the cryopreservation process (Wani et al., 2004b). Many studies have reported that the meiotic spindle of mature oocytes is extremely sensitive to low temperature (Aman and Parks, 1994; Chen et al., 2003; Wu et al., 2006; Gautam et al., 2008b; Morató et al., 2008; Attanasio et al., 2010). Exposure of bovine oocytes to 4°C for 10-20 min caused complete disappearance of the meiotic spindle (Aman and Parks, 1994). In general, the temperature that is used for storage of mammalian cells is -196°C (Jain and Paulson, 2006). When water is cooled to below its freezing point, the water exists in a crystalline structure known as ice which cause pressure and shearing force on intracellular organelles and lead to cell damage (Jain and Paulson, 2006). Recently, there are 2 methods for oocytes cryopreservation, slow freezing and vitrification.

Slow freezing is the standard method, which widely use in animal and human laboratories. This method requires an expensive programmable freezing machine (Figure 3) to control the cooling rates. Slow freezing procedures have many steps (Figure 4) : 1) the samples (oocyte or embryo) are exposed to cryoprotectants such as ethylene glycol (EG), DMSO, glycol or propylene glycol; 2) the samples are placed into programmable freezer and pre-cooled at -7°C, after three minutes of equilibration

at -7°C the samples are seeded to induce ice crystals; 3) the temperature is slowly decreased at around 0.3°C/min to -30°C; 4) the samples are then plunged into liquid nitrogen and storage at -196°C.

Figure 3. Programmable freezing machine

The whole freezing process of slow freezing takes about 2-3 h that is longer than vitrification (2-3 min) (Table 2). Slow freezing method uses cryoprotectants at low concentration which is less toxic to the cells but ice-crystals can be formed. This incident may cause DNA or intracellular organelles damage. Slow freezing has been less successful than vitrification in cyropreservation of matured buffalo oocyte (Gautam et al., 2008b). Compared to the higher proportion of oocyte developed to morula and blastocyst after vitrification than slow freezing (Gautam et al., 2008b). However, this technique does not require the specialist and the results have less variation when compared with vitrification method.

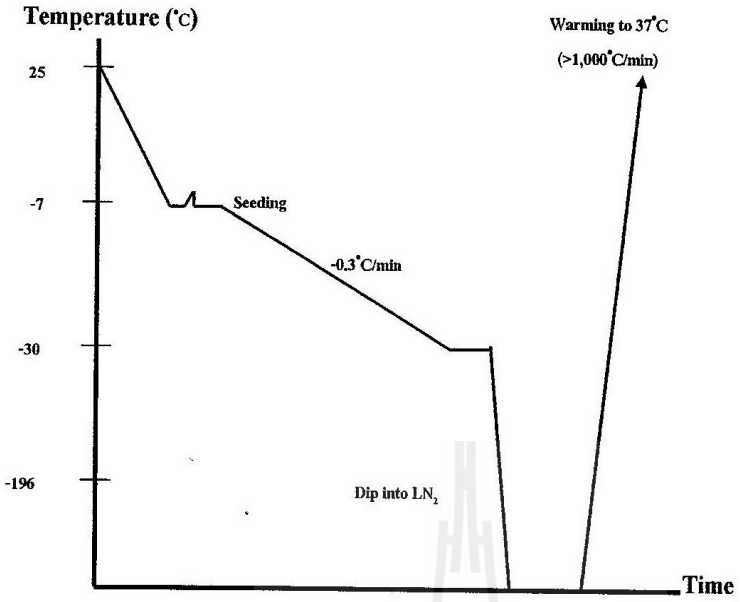

Figure 4. The cryopreservation procedure by slow freezing

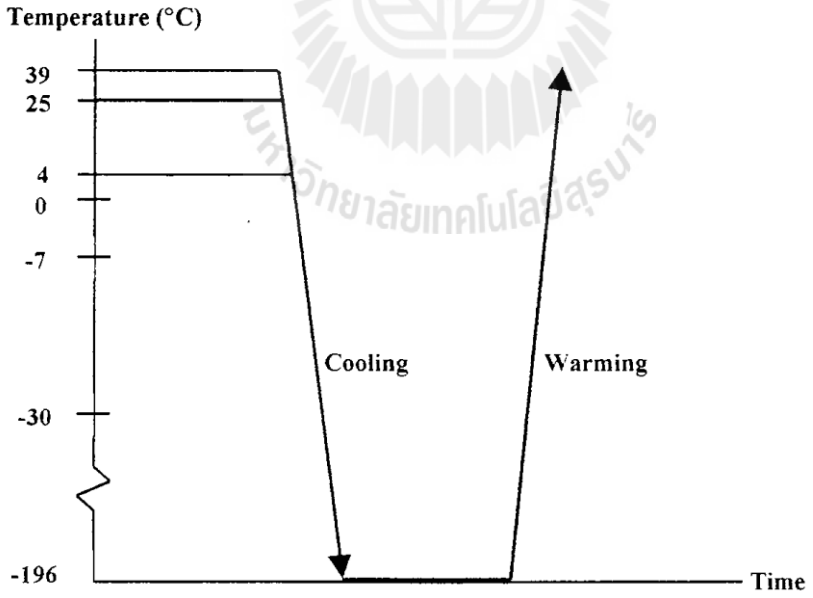

Figure 5. Cooling and warming rates of vitrification method

Table 2. Comparison of cryopreservation methods of slow freezing and vitrification

Vitrification	Slow freezing
1. No ice-crystal formation	1. Ice-crystal may be formed
2. No need expensive machine	2. Need programmable freezing machine
3. Use only 2-3 minutes in all process	3. Use 2-3 hours in all process
4. High concentration of cryoprotectants (~40% v/v)	4. Low concentration of cryoprotectants (~10% v/v)
5. Variation in results (depend on skill of technician)	5. Less variation in results (no need high skill of technician)

To avoid ice-crystals formation, the cryoprotectants at high concentration have been used. At high concentrations, the ice-crystal formation is inhibited and leads to the development of a glasslike which is called vitrified state (Jain and Paulson, 2006). Therefore, vitrification is the process of solidification of solution that has been developed over the years by using a high concentration of cryoprotectants to prevent the ice-crystals formation. However, the high concentration of cryoprotectants used for vitrification is toxic to the cells. Papis et al. (2000) found that pre-equilibration treatments of bovine oocytes in 6% EG showed lower cleavage rate than pre-equilibration of oocytes in 2% and 4% EG. Thus the cells can be exposed to high concentration for a short period of time or at very low temperatures (Jain and Paulson, 2006). The toxicity of cryoprotectants can be minimized by increasing the cooling rate with small volume of vitrification solution (Gasparrini et al., 2007) or directly contact

with liquid nitrogen (Chian et al., 2004). In addition, vitrification technique is a rapid and simple method, which uses only 2-3 min in all steps of freezing process. Vitrification has been applied to cryopreserve mammalian oocytes in many species including buffalo (Wani et al., 2004b; Gasparrini et al., 2007; Gautam et al., 2008b; Attanasio et al., 2010), bovine (Dinnyés et al., 2000; Chian et al., 2004; Checura and Seidel Jr, 2007; Morató et al., 2008; Sripunya et al., 2010), goat (Begin et al., 2003), mouse (Suo et al., 2008), ovine (Succu et al., 2007), hourse (Tharasanit et al., 2006), porcine (Wu et al., 2006; Somfai et al., 2006) and human (Kuwayama, 2007). However, this technology has had variation in the success rate, depending on the protocols used, laboratory technique and/or species.

Recently, several techniques which have been developed to improve the vitrification outcome such as open-pulled straws (OPS), microdrop, cryotop, cryoloop and solid surface vitrification (SSV). Bovine matured oocytes have been vitrified by using cryoloop and OPS, the results showed that higher cleavage (54.0 ± 3.5 vs. 48.9 ± 3.2) and blastocyst rates (10.8 ± 1.4 vs. 5.3 ± 1.3) were obtained with cryoloop method than OPS (Checura and Seidel Jr, 2007). In that study described, cryoloop allowed direct contact of the sample with liquid nitrogen with resulted in higher cooling and warming rates than OPS (Checura and Seidel Jr, 2007) which is one of the major goal of successful in cryopreservation. Subsequently, Morató et al (2008) reported that no blastocyst production from matured bovine oocyes vitrified with OPS, although the survival rate was high (88.4%). Moreover, matured calf oocytes vitrified by OPS showed significantly higher percentage of abnormal spindle structure (62.2%) when compared with control (30.1%), which associated with disorganized or decondensed microtubules or chromosomes (Morató et al., 2008). Aman and Parks

(1994) indicated that meiotic spindle in bovine oocytes did not fully re-form after re-warming, which may be limited by the amount of pericentriolar material available for organizing microtubule polymerization. However, the immature mouse oocytes have been successfully cryopreserved by OPS with high survival rate (94.1%), cleavage rates (55.7%) and blastocyst formation (40.9%) (Suo et al., 2008). The distinct results may be the reassembly of spindles displays species-specific differences after re-warming (Wu et al., 2006). Further study proved that SCNT blastocysts development (7.4%) was obtained from *in vitro*-matured oocytes vitrified by microdrop (Yang et al., 2008). To evaluate the complete developmental potential, embryo derived from vitrified-thawed oocytes were transferred to recipients, and two pregnancies were detected at day 60 and one of them lasted until day 222 (Yang et al., 2008).

One of the successful vitrification techniques is cryotop (Figure 6) vitrification. This technique has resulted in normal births derived from vitrified human oocytes than any other cryopreservation method (Kuwayama, 2007). Cryotop was also used for cryopreservation of bovine oocytes with high survival rate after warming but poor embryo developmental rates (Chian et al., 2004; Zhou et al., 2010). In another study, SSV (Figure 7) method has been reported to be highly efficient for bovine oocytes cryopreservation (Dinnyés et al., 2000). Subsequent development of SCNT embryos to cleavage (85% vs. 90%), blastocyst (27% vs. 29%), or hatched blastocyst stage (20% vs. 22%) in vitrified oocytes group did not statistically differ from control group (Dinnyés et al., 2000). Furthermore, Sripunya et al. (2010) proved that the cooling systems of the cryotop and SSV methods were equally effective for preservation of bovine oocytes in terms of their survival and subsequent development competence after IVF and IVC. Although, the survival rate of vitrified bovine oocytes

after warming was high (81-97.6%) but the embryo development to blastocyst stage after IVF was still low (0-12.8%) (Dinnyés et al., 2000; Chian et al., 2004; Checura and Seidel Jr, 2007; Yang et al., 2008; Sripunya et al., 2010; Zhou et al., 2010). Men et al. (2003) investigated the degeneration mechanism of cryopreserved oocytes. They reported that the biochemical markers of apoptosis, such as DNA fragmentation and activation of caspases, were detected not only oocytes having typical apoptotic morphology, but also in oocytes without observable apopototic morphology. Accordingly, the freezing frequency caused the zona pellucida to rubture, cumulus cells to detach, and gap junctions between cumulus cells and oocyte to be interrupted which influencing subsequent development and maturation of oocytes (Wu et al., 2006). Nevertheless, the high lipid content in oocyte cytoplasm is still one of the major causes in poor embryo development of porcine and buffalo oocytes following vitrification, because high amount of lipid droplets make the oocyte more sensitive to chilling injury (McEvoy et al., 2000; Fujihira et al., 2004; Wu et al., 2006; Boonkusol et al., 2007; Gasparrini et al., 2007; Attanasio et al., 2010). Recently, Somfai et al. (2011) found that the density of lipid droplets was reduced when cultured the oocytes in IVM medium supplementation with L-carnitine. Thus pre-treated bovine oocytes with L-carnitine following vitrification may provide a new technique for improvement the oocyte cryopreservation, which may lead to cryoinjury reduction by way of the lipid droplets decrease.

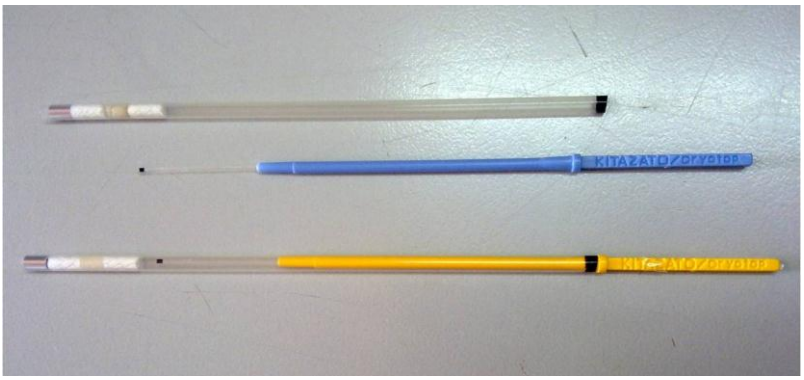

Figure 6. The cryotop vitrification device (Kitazato Supply Co., Tokyo, Japan)

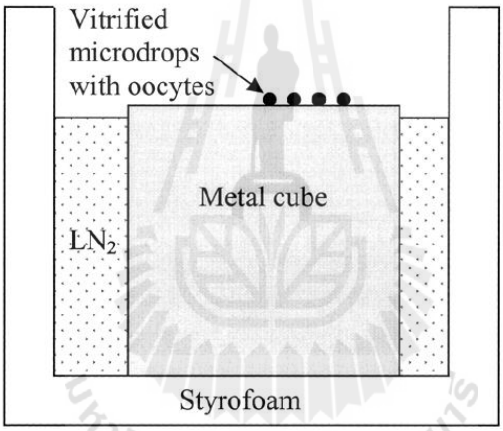

Figure 7. The solid surface vitrification (SSV) device (Dinnyés et al., 2000)

2.5 Lipid in mammalian oocytes

Oocytes of all mammals contain an endogenous lipid reserve (McEvoy et al., 2000). Intracellular lipids play a significant metabolic role as energy source for protein synthesis which supports the cytoplasmic and nuclear maturation of the oocytes (Sturmey and Leese, 2003). In addition, lipid plays an important role in cell structure and in modifying the physical properties and metabolic function of biological membranes (Kim et al., 2001). Studies in lipids on porcine (Cran, 1985; Nagashima et

al., 1994; Isachenko et al., 1998; Sturmey and Leese, 2003; Fujihira et al., 2004), bovine (Isachenko et al., 2001; Kim et al., 2001; Leroy et al., 2005; Ferguson and Leese, 2006) and sheep oocytes (McEvoy et al., 2000) have examined the fatty acid composition and triglyceride content. The lipid content of oocytes and embryos is an important parameter linked to quality and cryotolerance (Leroy et al., 2005). Porcine oocytes and early embryos had a dark color tone of cytoplasm, due to a high quantity of endogenous lipid. Didion et al. (1990) reported that porcine oocytes did not survive after cooling to lower than 15 degrees, and the *in vitro* maturation rates of frozen-thawed porcine oocytes were significantly lower than those of other species. Several studies have suggested that the sensitivity of porcine oocytes/embryos to low temperature may relate to their relatively high intracellular lipid content (Nagashima et al., 1994; McEvoy et al., 2000; Fujihira et al., 2004). Bovine oocytes also contain a large number of lipid droplets (63 ± 6 µg) in cytoplasm (McEvoy et al., 2000). It was reported that bovine oocytes are also sensitive to low temperature (Aman et al., 1994). It has been shown that 56% of oocytes exposed to 25°C and 90% of oocytes exposed to 4°C for 1 min had abnormal spindle (Aman et al., 1994). Martino et al. (1995) reported that the developmental rate of bovine GV oocytes is also impaired after exposure to 10°C for 30 min or to 0°C for only 30 sec. Isachenko et al. (1998) suggested that the freeze/thaw process can induce an alteration in the physicochemical properties of intracellular lipids. Additionally, Isachenko et al. (2001) supposed that the negative effect of cooling on oocytes may be explained by the effect of cooling lipids on cytoskeleton structures. Lipid droplets interact with the elements of cytoskeleton in the oocytes (Fujihira et al., 2004). These interactions between the lipid phase of cells and the elements of the cytoskeleton are complex and hardening of

these lipids might cause deformation and disruption of the cytoskeleton (Isachenko et al., 1998). Moreover, it was reported that the cytoplasmic membrane is the major organelle sensitive to low temperature (Schmidt et al., 1993; Arav et al., 1996). Phospholipid and cholesterol are essential for the formation of membranes, which are a crucial requirement during the rapid cell divisions after fertilization (McEvoy et al., 2000). The physical properties of this membrane are influenced by lipid composition and regulated in response to environmental factors (Quinn, 1989). Previous studies revealed that the changes in lipid content (mainly phospholipid) were observed in frozen-thawed immature bovine oocyes (Kim et al., 2001). The change in phospholipid may lead to a decrease in membrane fluidity and reduced ability to tolerate low temperature (Kim et al., 2001). In 1994, Nagashima et al. found that the porcine embryos gain tolerance to chilling when their lipid content is reduced. Therefore, the survivability of oocytes after freezing and thawing could be enhanced by improving the cryopreservation method which causing a decrease in the lipid contents of oocytes (Fujihira et al., 2004).

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CHAPTER III

MATERIALS AND METHODS

3.1 Experimental designs

Experiment 1 was performed to study the effect of L-carnitine in IVM medium on nuclear maturation. Cumulus oocyte complexes (COCs) were randomly cultured in IVM medium supplemented with 0.3, 0.6, and 1.2 mg/mL of L-carnitine (0.3, 0.6 and 1.2 groups, respectively). IVM medium with no addition of L-carnitine, served as a control group (0 mg/mL). The nuclear stage of oocytes was assessed by fixing and staining with aceto-orcein dye.

Experiment 2 was performed to evaluate the effect of L-carnitine in IVM medium on developmental ability of embryos after fertilized by IVF. After IVM, the oocytes in each group were fertilized with fertile frozen-thawed sperms. The embryo developmental competence was compared to those of L-carnitine-treated and control group.

Experiment 3, 0.6 mg/mL of L-carnitine (based on the results of Experiment 1 and 2) was used to investigate the effects of L-carnitine for IVM of bovine oocytes on their cryopreservation. After thawing, only survival oocytes were incubated with fertile frozen-thawed sperms and embryo developmental competence *in vitro* was compared to those of L-carnitine-treated and control groups.

Experiment 4, 0.6 mg/mL of L-carnitine (based on the results of Experiment 1 and 2) was used to evaluate the effects of L-carnitine in IVF and IVC medium on

developmental competence of bovine embryos. The developmental competence of embryos to blastocyst stage was compared between L-carnitine-treated and control group.

3.2 Oocyte collection

Slaughterhouse bovine ovaries (Figure 8A) were transported to the laboratory in saline solution (0.9% NaCl) at room temperature. The COCs (Figure 8B) were collected from 2-8 mm follicles using 18-G needle connected with 10 mL syringe. The COCs were washed five times in modified Dulbecco's phosphate buffered saline (mDPBS) supplemented with 0.1% polyvinyl pyrolidone (PVP, Sigma, P-0930) to remove residual blood and follicular fluid.

3.3 *In vitro* maturation of oocytes

Each of 20 COCs was randomly cultured in 100 µL IVM medium supplemented with L-carnitine (C0158, Sigma) at 0, 0.3, 0.6, and 1.2 mg/mL (control, 0.3, 0.6 and 1.2 group, respectively) at 38.5 °C under humidified atmosphere of 5% CO_2 in air for 23 h (Figure 8C). The IVM medium was TCM-199 supplemented with 10% fetal bovine serum (FBS), 0.02 AU/mL follicle stimulating hormone (FSH, Antrin®, Denka Phamaceutical Co., Kanagawa, Japan), 50 IU/mL human chorionic gonadotropin (hCG, Chorulon®, Intervet UK Ltd) and 1 µg/mL 17β estradiol. (E8875, Sigma).

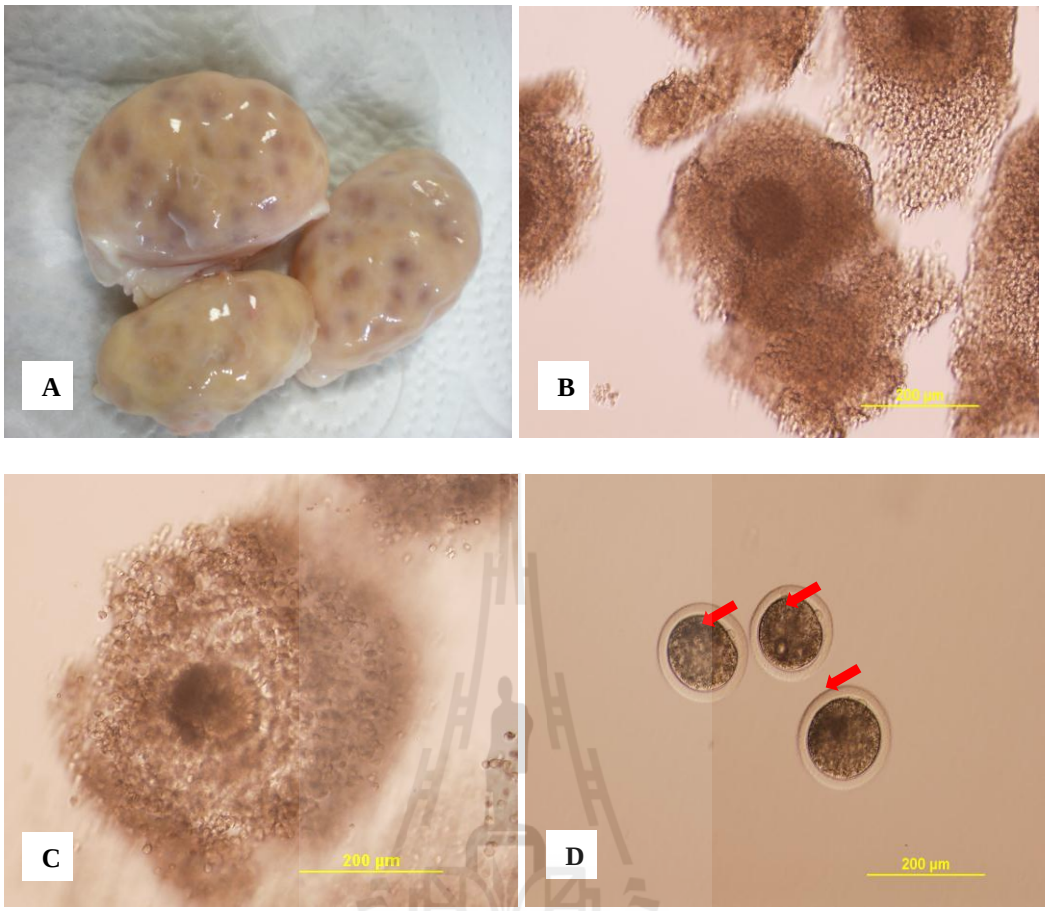

Figure 8. Oocyte collections. Bovine ovaries (A) from slaughter house were transported to laboratory. Oocytes with compact cumulus cells after manually aspirating (B). Oocyte with expanded cumulus cells after 23 h of IVM (C). Denuded oocytes after IVM extruded first polar body (arrow) (D, magnification 100x)

3.4 Oocyte vitrification and warming

After IVM, COCs were partially denuded by gentle pipetting with a pulled-pipette using 0.1% (w/v) hyaluronidase. A group of five oocytes were placed in equilibration solution containing 10% dimethylsulfoxide (DMSO, MERCK, Darmstadt, Germany) and 10% ethylene glycol (EG) in TCM199-Hepes + 20% FBS (BM) for 1 min and then exposed to vitrification solution containing 20% DMSO, 20% EG and 0.5 M sucrose in BM for 30 sec, at 22 to 24 °C. Oocytes were loaded onto the end tip of cryotop (Kitazato Supply Co., Tokyo, Japan) in a small volume of vitrification solution and plugged into liquid nitrogen immediately. After seven days, vitrified-oocytes from liquid nitrogen were directly immersed into 3 mL of BM supplemented with 0.5 M sucrose at 38.5 °C for 5 min, and then transferred into BM for 5 min. Finally, the vitrified-warmed oocytes were kept in BM under humidified atmosphere of 5% CO_2 in air at 38.5 °C for 1 h before being used.

3.5 Evaluation of oocyte viability

The oocyte viability was determined by fluorescein diacetate (FDA, F7378, Sigma) staining. Briefly, oocytes were treated with 2.5 µg/mL FDA in phosphate buffered saline (PBS) supplemented with 5 mg/mL bovine serum albumin (BSA) at 38.5 ºC for 2 min in dark room, and then washed three times in PBS supplemented with 5 mg/mL BSA. The oocytes were evaluated under UV light provided by a 100W high-pressure mercury burner (Olympus, Tokyo, Japan) for fluorescence microscope. Oocytes expressing a bright green fluorescence (Figure 9B) were regarded as living and were used in subsequent experiments.

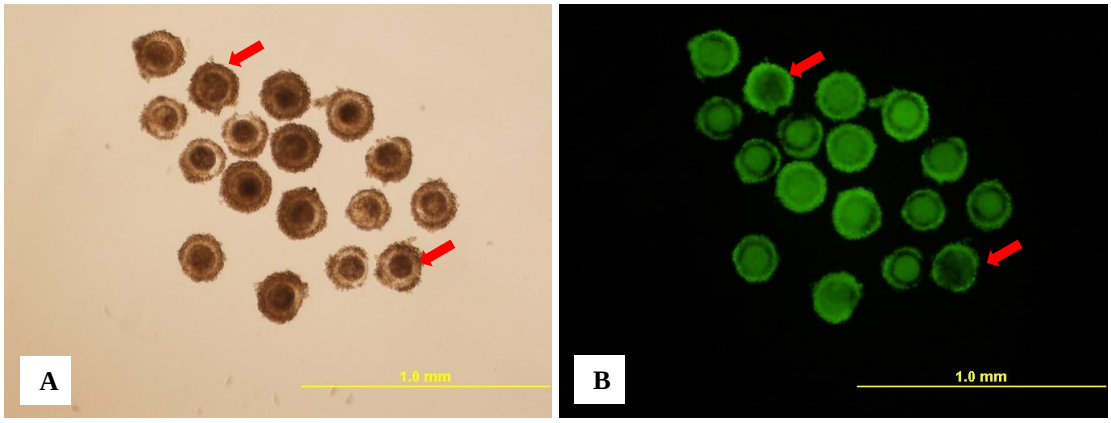

Figure 9. FDA stained vitrified bovine oocytes in bright field (A) and fluorescence image (B). Oocytes expressing a bright green fluorescence were regarded as living; arrow indicated the dead oocytes following vitrification and FDA staining (magnification 40x)

3.6 *In vitro* fertilization (IVF)

The frozen bull sperm was thawed in air for 10 sec and placed in 37 °C water bath for 30 sec. Thawed sperm was placed in the bottom of 5 mL snap tube containing 2 mL of TALP (Tyrode's albumin lactate pyruvate) medium and incubated at 38.5 °C under humidified atmosphere of 5% CO_2 in air for 30 min to allow them to swim up. The sperm suspension (1.5 mL) was collected from the top of the tube and centrifuged at 400 x g for 5 min. The pellets were re-suspended in TALP medium and washed twice at 400 x g for 5 min. Groups of 10-15 COCs were placed in 100 µL droplets of TALP medium, and incubated with frozen-thawed sperm at a concentration of 3 x 10^6 /mL at 38.5 °C under humidified atmosphere of 5% CO_2 in air for 12 hours. In Experiment 4, the COCs in 0.6 mg/mL group were incubated with frozen-thawed sperm in TALP medium supplemented with L-carnitine at 0.6 mg/mL.

3.7 *In vitro* culture (IVC)

The embryos (20 embryos/100 µL) were cultured in mSOFaa medium supplemented with 3 mg/mL fatty acid free BSA (A-6003, Sigma) at 38.5 °C in 5% CO_2, 5% O_2 and 90% N_2 for 2 days. Only 8 cell stage embryos (Figure 10A) were co-cultured with bovine oviductal epithelium cells in mSOFaa medium supplemented with 0.25 mg/mL glucose under humidified atmosphere of 5% CO_2 in air for 2 days. After that, the embryos were cultured in mSOFaa medium supplemented with 0.5 mg/mL glucose for an additional 3 days (7 days total). In Experiment 4, the COCs in 0.6 mg/mL group were cultured in mSOFaa medium supplemented with 0.6 mg/mL of L-carnitine until blastocyst stage (Figure 10B) (7 days; the day of IVF was regarded as Day 0).

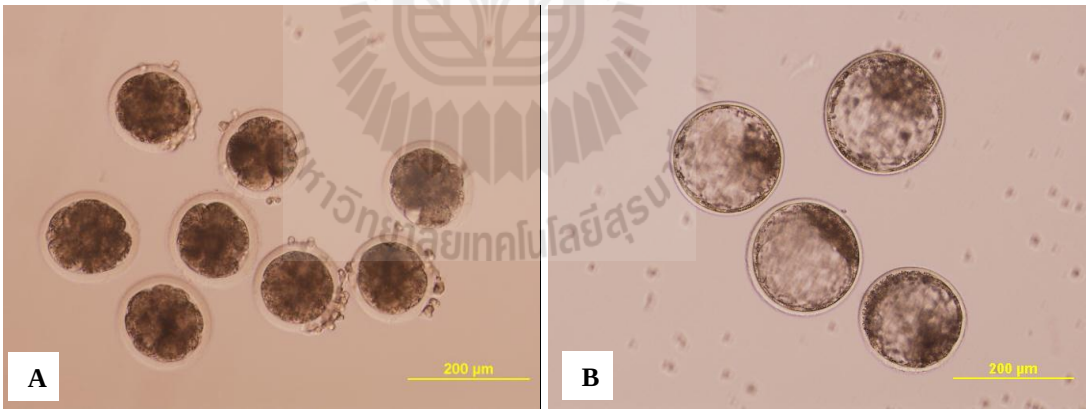

Figure 10. Embryo development of bovine at 8 cell (A) and blastocyst (B) stage after *in vitro* culture for 2 and 7 days, respectively (magnification 100x)

3.8 Evaluation of nuclear maturation

After IVM, COCs were treated with 0.1% (w/v) hyaluronidase to remove cumulus cells by gently using a fine-tip pipette. Oocytes were mounted on a glass slide and fixed with ethanol: acetic acid (3:1). After three days of fixation, oocytes were stained with 1% (w/v) orcein in acetic acid for 10 min, and rinsed with glycerol: acetic acid: water (1:1:3) to remove the residual dye. The nuclear stage was evaluated under a phase-contrast microscope (Olympus, Tokyo, Japan). Oocytes displaying a metaphase plate and one pink polar body (PB) were deemed to be at the metaphage-II stage (MII) (Figure 11).

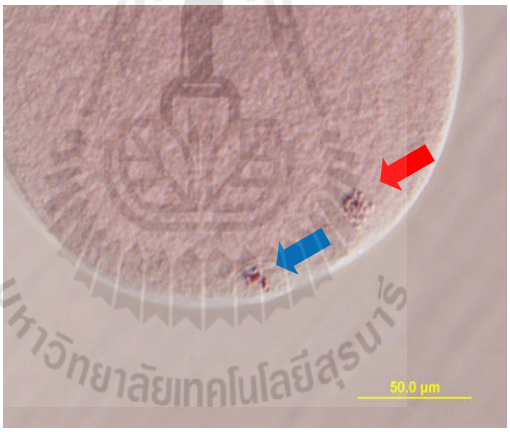

Figure 11. Metaphase-II stage (MII stage) oocyte after stained with aceto-orcein dye showed metaphase plate (red arrow) and first polar body (blue arrow) in pink color (magnification 400x)

3.9 Evaluation of blastocyst quality

The evaluation of blastocyst quality was assessed using the method described by Thouas et al. (2001). Briefly, the blastocysts were treated with 0.1 mg/mL propidium iodide (PI) and 0.2% Triton X-100 dissolved in DPBS for 1 min. Then the blastocysts were incubated with 25 µg/mL Hoechst 33342 (Calbiochem, San Diego, CA, USA) dissolved in 99.5% ethanol for 5 min. The blastocysts were mounted on glass slides in glycerol droplets. The numbers of trophectoderm (TE) and inner cell mass (ICM) were counted under UV light provided by a 100W high-pressure mercury burner (Olympus, Tokyo, Japan) for fluorescence microscope to determine the quality of blastocysts. From these, the ICM showed blue color of Hoechst and TE showed pink or red color of both PI and Hoechst (Figure 12).

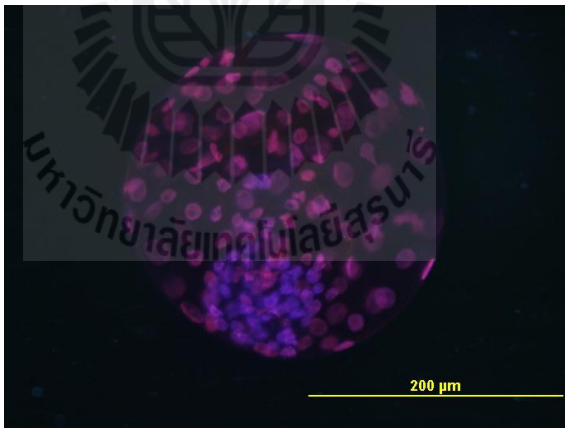

Figure 12. Fluorescence image of bovine blastocyst. Blue color of Hoechst stained inner cell mass (ICM) and pink or red of both PI and Hoechst stained trophectoderm (TE) cells (magnification 200x)

3.10 Statistical analysis

Each treatment was repeated at least five times. Data for nuclear maturation, embryo development, survival and number of TE and ICM cells were arcsine transformed and analyzed by ANOVA using statistical analysis systems (SAS). *P* value at < 0.05 was considered a statistically significant difference.

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Experiment 1 : Effects of L-carnitine addition to IVM medium on maturation rate of bovine oocytes

After 23 hours of IVM, all oocytes from L-carnitine (0.3, 0.6 and 1.2 mg/mL of Lcarnitine) and non L-carnitine (0 mg/mL of L-carnitine; control group) treatment groups were denuded to remove the cumulus cells. Then, the maturation rates of bovine oocytes were evaluated by fixing and staining with aceto-orcein dye. The MII stage oocytes showed the metaphase plate and first polar body in red color and percentage of MII was recorded. In the present study, L-carnitine supplementation to IVM medium improved maturation rates of bovine oocytes (Table 3). The highest maturation rates were obtained in the 0.3 and 0.6 groups (77.1% and 77.9%, respectively) as they were significantly higher than that in the control group (67.8%). This finding coincides with the results of Somfai et al. (2011) reporting that the maturation rate of porcine oocytes was increased in IVM medium supplemented with 0.6-5 mg/mL L-carnitine compared with no addition. In contrast, our results are different from that reported by Wu et al. (2011) who also studied the effects of L-carnitine in porcine species. Their results showed that oocyte maturation rate in L-carnitine (0.25-1 mg/mL) group did not differ from that in control group (no addition); however, the maturation rate of porcine oocytes was even reduced when supplemented with 2 mg/mL L-carnitine. In our study, although the maturation rate in

the 1.2 mg/mL group (75.8%) appeared higher, it was not significantly different when compared with the control group (67.8%). These conflicting results may be due to differences in IVM conditions, IVM procedures and/or different species used.

The different respondence between bovine and pocine oocytes might be explained by the differences in kinetics of nuclear maturation between bovine and porcine oocytes (Wehrend and Meinecke, 2001). In average, porcine oocytes remained 23.4 h in germinal vesicle (GV) stage, 9.6 h in metaphase I (MI), 2.8 h in anaphase I (AI) and 1.9 h in telophase I (TI). In bovine, oocytes remained 8.5 h in GV stage, 8.3 h in MI, 1.6 h in AI and 1.9 h in TI. In porcine, MI stage can be observed between 26 and 34 h, AI and TI between 29 and 46 h and MII after 35 h of cultivation. On the other hand, MI stage of bovine oocyte was observed from 10 – 18 h and AI and TI from 16 – 24 h after cultivation. From above result, it indicated that the duration of meiotic stages differ between species (Wehrend and Meinecke, 2001). In addition, the difference between bovine and porcine oocyte during maturation not only the length of meiotic stage but also the M-phase promoting factor (MPF) and mitogen-activated protein kinase (MAP kinase) activities. The MAP kinase in porcine oocytes was activated before germinal vesicle breakdown (GVBD) and MPF activation (Wehrend and Meinecke, 2001) and also induces GVBD (Inoue et al., 1998). For bovine oocytes, the MAP kinase and MPF were activated at approximately the same time as the GVBD (Fissore et al., 1996; Wehrend and Meinecke, 2001). Therefore, the conflicting results in bovine and porcine oocyte maturation in response to L-carnitine treatment during IVM may be due to the difference in kinetics of meiotic progression, MPF and MAP kinase activities.

Table 3. Effects of L-carnitine addition to IVM medium on maturation rate of bovine oocytes

Groups	No. oocytes	No. (%) of MII oocytes
0 mg/mL	149	101/149 (67.8) [b]
0.3 mg/mL	153	118/153 (77.1) [a]
0.6 mg/mL	154	120/154 (77.9) [a]
1.2 mg/mL	153	116/153 (75.8) [ab]

Five replicates were performed.

Different superscripts within column indicate significant differences ($P < 0.05$).

4.2 Experiment 2 : Effects of L-carnitine addition to IVM medium on embryo developmental competence of bovine oocyte after IVF

After IVM, when the oocytes were subjected to IVF and continued to be cultured *in vitro*, the cleavage rates were similar among L-carnitine groups and control group (Table 4). The highest 8-cell, morula and blastocyst rates were obtained in 0.6 mg/mL of L-carnitine group compared with other groups. Similar results were obtained by Wu et al. (2011). When their porcine oocytes were treated with 0.5 mg/mL of L-carnitine during IVM, there were significantly greater rates of blastocyst formation than that of control (no addition). However, in the present study, there was no significant difference in TE and ICM cell numbers among those groups (Table 5). Therefore, our results suggest that supplementation of L-carnitine at 0.6 mg/mL

during IVM is most suitable for bovine oocyte maturation in terms of the highest embryo developmental rates obtained compared with other groups.

The improvements of oocyte maturation and embryo development by supplementation of L-carnitine may result from the utilization of lipid via β-oxidation to generate the ATP, which is necessary for the resumption of meiosis and cytoplasmic maturation (Ferguson and Leese, 2006). It was also reported that L-carnitine supplementation during oocyte maturation significantly increased β-oxidation, and improved both fertilization and blastocyst developmental rates (Dunning et al., 2010; Dunning et al., 2011). Moreover, enhancing mitochondrial functions by using L-carnitine improved oocyte maturation, underlining the importance of lipid metabolism for nuclear and cytoplasmic maturation of oocytes (Somfai et al., 2011). Additionally, it was revealed that bovine oocytes use endogenous lipid as a key energy source during maturation (Cetica et al., 2002; Ferguson and Leese, 2006). When methyl palmoxirate (a fatty acid oxidation inhibitor) was added to the maturation medium of bovine oocytes, the cleavage and blastocyst formation rates and cell numbers were reduced (Ferguson and Leese, 2006). This suggested that endogenous lipid metabolism plays an important role during oocyte maturation and potentially in subsequent embryonic development.

Besides its role in metabolism, L-carnitine acts as an antioxidant reducing the levels of ROS in oocytes during IVM (Wu et al., 2011; Somfai et al., 2011). Incubation of oocytes and embryos with L-carnitine was reported to reduce cytoskeleton damage and decrease the level of apoptosis via its antioxidant action (Mansour et al., 2009). Since both lipid metabolism and redox status are important for the maturation and further competence of oocytes, the present study proposes that the

improvement of maturation and their subsequence embryonic development after IVF of bovine oocytes by treatment with L-carnitine may either be due to (1) its action as an enhancer of lipid metabolism via β-oxidation to generate the ATP which is necessary for oocyte metabolism; and/or (2) it acts as an antioxidant (ROS scavenger) by preventing apoptotic events in oocytes (Mansour et al., 2009) and embryonic cells (Abdelrazik et al., 2009) through its antioxidant effect.

On the other hand, present results found that bovine embryonic development to the blastocyst stage was reduced when IVM in the supplementation of 1.2 mg/mL of L-carnitine. Similar result were obtained by Wu et al. (2011), the blastocyst rate was decreased when porcine oocytes were exposed to 2 mg/mL of L-carnitine during IVM. This incident can explicate that the reduction of bovine blastocyst formation rate in 1.2 mg/mL group may be due to the high concentration of L-carnitine, which could induce an imbalanced high amount of ATP production. The excessively high levels of ATP in matured oocytes may indicate an impaired developmental capacity and disruption of oocyte functions for consuming ATP (Nagano et al., 2006b). Previous studies reported that the oocytes with a high level of ATP showed lower rates of maturation and poorer in cleavage and embryo development to blastocyst stage when compared with the oocytes had moderate levels of ATP (Nagano et al., 2006a; Nagano et al., 2006b). More importantly, the storage of ATP at proper level in matured oocytes is one of the key factors determining subsequent embryonic development and the quality of resulting blastocyst (Nagano et al., 2006b). However, the actions of L-carnitine need to be further clarified on maturation and embryonic developmental competence of bovine oocyte.

Table 4. Effects of L-carnitine addition to IVM medium on embryo developmental rate of bovine oocytes after IVF

Groups	Cultured	Cleaved (%)	No. (%) embryo developed to		
			8 Cell	Morula	Blastocyst
0 mg/mL	141	104 (73.8)	54 (38.3) [b]	38 (27.0) [b]	33 (23.4) [b]
0.3 mg/mL	138	104 (75.4)	54 (39.1) [b]	41 (29.7) [ab]	33 (23.9) [b]
0.6 mg/mL	144	113 (78.5)	67 (46.5) [a]	45 (31.3) [a]	42 (29.7) [a]
1.2 mg/mL	140	102 (72.9)	47 (33.6) [b]	35 (25.0) [b]	26 (18.6) [c]

Five replicates were performed.

Different superscripts within column indicate significant differences ($P < 0.05$).

Table 5. Effects of L-carnitine addition to IVM medium on bovine blastocyst quality

Groups	N	Mean (± S.D.) number of cells in blastocyst			
		TE	ICM	Total cells	ICM ratio (%)
0 mg/mL	20	76.0 ± 13.2	22.8 ± 5.1	98.8 ± 13.2	23.3 ± 5.2
0.3 mg/mL	20	90.5 ± 11.3	29.1 ± 5.8	118.3 ± 14.4	24.3 ± 4.2
0.6 mg/mL	20	91.2 ± 7.3	29.7 ± 5.4	120.5 ± 9.7	24.5 ± 3.2
1.2 mg/mL	20	90.3 ± 14.5	25.8 ± 6.6	116.9 ± 21	22.0 ± 3.5

Five replicates were performed.

The data was not statistically difference ($P > 0.05$).

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4.3 Experiment 3 : Effects of L-carnitine addition to IVM medium on survival rate of vitrified-warmed bovine oocyte and subsequent embryo developmental competence after IVF

Based on the results of Experiment 1 and 2, the optimum concentration of L-carnitine during bovine IVM was 0.6 mg/mL. Thus in Experiment 3, 0.6 mg/mL concentration was used to investigate the effects of L-carnitine addition to IVM of bovine oocytes on their cryopreservation. High survival rates were achieved from vitrified bovine oocytes irrespective of L-carnitine treatment (with L-carnitine 88.2% and without L-carnitine 85.7%, Table 6). These survival rates are similar to those of previous reports using the Cryotop method to vitrify bovine oocytes (Chian et al., 2004; Morató et al., 2008; Sripunya et al., 2010). In the present study, after warming, only surviving oocytes were subjected to IVF and continued to be cultured *in vitro* until the blastocyst stage. Interestingly, the ability of embryos to develop to the 8-cell stage in the 0.6 mg/mL of L-carnitine group after vitrification (0.6 Vitri group) was not significantly different from the control group without L-carnitine treatment and vitrification (33.94% and 41.62%, respectively). It is possible that L-carnitine can improve oocyte quality (Mansour et al., 2009) which would suggest their improved tolerance to cryo-damage that resulted in high survival rate of oocyte and their subsequent development to early stage after vitrification. However, the cleavage, morula and blastocyst rates were not significantly different between vitrified groups and additionally, the TE and ICM cells of blastocysts in vitrified groups were not significantly different and were lower than those of fresh groups irrespective of L-carnitine treatment (Table 7). Present results suggested that L-carnitine did not affect bovine oocytes cryopreservation outcome.

The lipid content of oocytes and embryos is an important parameter linked to quality and cryotolerance (Leroy et al., 2005). Lipids play a significant role in energy storage, but on the other hand, they are a "stumbling block" during oocyte cryopreservation (Liebermann et al., 2002). Porcine and buffalo oocytes are supposed to be particularly sensitive to chilling injuries, because of their high intracellular lipid content in their cytoplasm (McEvoy et al., 2000; Fujihira et al., 2004; Wu et al., 2006; Gasparrini et al., 2007; Attanasio et al., 2010). The study from Nagashima et al. (1994) clearly showed that porcine embryos became tolerant to chilling when their lipid content was reduces. Bovine oocytes also have intracytoplasmic lipid contents (McEvoy et al., 2000; Shaw et al., 2000; Kim et al., 2001), therefore the survivability of oocytes after freezing and thawing could be enhanced by improving the cryopreservation method causing a decrease in the lipid content of oocytes (Fujihira et al., 2004). Recently, lipid droplets of porcine oocytes were decreased when oocytes were cultured in IVM medium supplemented with L-carnitine (Somfai et al., 2011). Our study hypothesized that, vitrified bovine oocytes following L-carnitine pre-treatment may lead to reduction of cryoinjury by way of decrease in lipid droplets. However, results from the present study indicated that supplementation of L-carnitine during IVM had no positive effects on survival rate, blastocyst formation and blastocyst quality of bovine oocytes after vitrification. Bovine oocytes were reported to be more considerably cryostable than porcine oocytes (Liebermann et al., 2002). Isachenko et al. (2001) compared the effect of cooling on ultrastructure of intracellular lipid droplets between bovine and porcine GV oocytes, and revealed that lipid droplets in porcine oocytes changed morphologically during cooling and they turned shape from round into spherical form. In contrast, lipid droplets in bovine oocytes revealed

that no morphological changed after cooling (Isachenko et al., 2001). The changes in lipid droplet after freezing could disrupt the connection between mitochondria and lipid droplets might affect developmental competence of oocytes (Wu et al., 2006). Moreover, the lipid droplets of bovine oocytes have a homogenous structure, whereas porcine oocytes have two kinds, dark and gray vesicle of lipid droplets (Isachenko et al., 2001). In addition, the mean (± SEM) fatty acid content of porcine oocytes was 161 ± 18 µg, approximately 2.5 fold the amount of bovine oocytes (63 ± 6 µg) (McEvoy et al., 2000). These incidents might be explained by the lipid is species-specific difference in terms of its apparent and utilization, may underlie the contrasting chilling and cryopreservation sensitivities of oocytes derived from different species (McEvoy et al., 2000). However, the relationship among L-carnitine, lipid droplets and cryosensitivities of bovine oocytes after cryopreservation need to be further investigated.

Moreover, the results of present study showed that the embryo developmental rates in vitrified groups were not significantly different irrespective of L-carnitine treatment. This might be due to various damages caused by vitrification, such as zona pellucida fracture, gap junction disruption between cumulus cells and oocytes (Wu et al., 2006), and spindle, chromosomes structure abnormality (Morató et al., 2008). In addition, DNA fragmentation and caspase-3 activity, which is a biochemical marker of apoptosis, were detected not only in oocytes having typical apoptotic morphology, but also in oocytes without observable apoptotic morphology after warming and culture (Men et al., 2003). It is possible that these cryodamages may reduce or neutralize the positive effects of L-carnitine on developmental capacity of cryopreserved bovine oocytes. This present study proposed that L-carnitine might be not effective enough to

overcome the damages caused by vitrification. Although, the blastocyst rate from vitrified groups in our study were similar to that reported by Sripunya et al. (2010), but higher than those reported by Morató et al. (2008) and Zhou et al. (2010) which used the cryotop method for *in vitro* matured bovine oocytes vitrification.

4.4 Experiment 4 : Effects of L-carnitine addition to IVM, IVF and IVC medium on embryo developmental competence

When oocytes were exposed to L-carnitine in all steps of culture including IVM, IVF and IVC, there was no significant difference in rates of 8-cell stage, morula and blastocyst formation, compared with non L-carnitine-treated group (Table 8). Also, the number of TE and ICM cells in the L-carnitine-treated group was not different from the non L-carnitine-treated group (Table 9). Although, the cleavage rate in the L-carnitine-treated group (67.8%) appeared lower, it was not significantly different when compared with the non L-carnitine-treated group (80.6%, $P>0.05$). One probable reason to describe this phenomenon is via antioxidant activity of L-carnitine. Previous studies found that the addition of antioxidants at high concentration in IVF medium significantly decreased the cleavage rates and the proportion of oocytes developed to blastocysts (Ali et al., 2003). Recently, it was reported that L-carnitine has an antioxidant activity by reducing the levels of ROS (Abdelrazik et al., 2009; Wu et al., 2011; Somfai et al., 2011) which might play a positive role during IVF (Ali et al., 2003). ROS at low and controlled concentration is required for hyperactivation, capacitation and acrosome reaction of human sperm (Aitken and Fisher, 1994; de Lamirande et al., 1997). In addition, the increased concentration of antioxidants significantly reduced the penetration rate of bovine sperm (Blondin et al., 1997). It is

suggested that ROS may play a crucial role for fusion of sperm and oocyte (Blondin et al., 1997; Ali et al., 2003). Thus, the reduction of cleavage rate in L-carnitine treated groups might be explained by the decrease of the ROS level which is necessary for sperm function and fusion through antioxidant effect of L-carnitine.

Moreover, the present results suggest that L-carnitine addition during the entire IVM/IVF/IVC procedure is not advantageous. Nevertheless, research on mouse has confirmed that addition of L-carnitine in the embryo culture medium can improve both percentages of blastocyst developmental rate and quality of the embryo (Abdelrazik et al., 2009). The discrepancy between these two studies might be attributed to *in vitro* embryo development period difference between mouse and bovine. Additionally, the oviduct epithelium cells co-cultured with bovine embryos might neutralize the effects of L-carnitine during IVC procedure. However, the present study is the first report in the effect of L-carnitine addition during the entire IVM/IVF/IVC procedure on developmental competence of bovine oocyte, therefore further investigation on this finding still needs to be further clarified.

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Table 6. Effects of L-carnitine addition to IVM medium on survival and embryo developmental rate of vitrified-warmed bovine oocytes after IVF

Groups		Survived (%)	Cultured	Cleaved (%)	No. (%) embryo developed to		
					8 Cell	Morula	Blastocyst
0	Fresh	173/173 (100)	173	134 (77.5) [a]	72 (41.6) [ab]	56 (32.4) [a]	42 (24.3) [b]
0	Vitri	166/194 (85.57)	166	111 (66.9) [b]	51 (30.7) [c]	35 (21.1) [b]	18 (10.8) [c]
0.6	Fresh	172/172 (100)	172	131 (76.2) [a]	81 (47.1) [a]	63 (36.6) [a]	53 (30.8) [a]
0.6	Vitri	165/187 (88.24)	165	113 (68.5) [b]	56 (33.9) [bc]	40 (24.2) [b]	22 (13.3) [c]

Five replicates were performed.

Different superscripts within column indicate significant differences ($P < 0.05$).

Table 7. Effects of L-carnitine addition to IVM medium on bovine blastocyst quality derived from vitrified oocytes

Groups	N	Mean (± S.D.) number of cells in blastocyst			
		TE	ICM	Total cells	ICM ratio (%)
0 Fresh	15	94.1 ± 13.8 [a]	28.1 ± 6.6 [a]	122.2 ± 14.1 [a]	23.1 ± 5.3 [a]
0 Vitri	15	78.6 ± 6.7 [b]	19.5 ± 6.0 [b]	98.1 ± 12.4 [b]	19.5 ± 3.3 [b]
0.6 Fresh	15	105.0 ± 10.4 [a]	30.5 ± 6.7 [a]	135.5 ± 10.1 [a]	22.6 ± 4.6 [ab]
0.6 Vitri	15	80.3 ± 6.4 [b]	21.8 ± 3.4 [b]	102.1 ± 6.9 [b]	21.4 ± 3.1 [ab]

Five replicates were performed.

Different superscripts within column indicate significant differences ($P < 0.05$).

Table 8. Effects of L-carnitine addition to IVM, IVF and IVC medium on embryo developmental rate of bovine oocytes

Groups	Cultured	Cleaved (%)	No. (%) embryo developed to		
			8 Cell	Morula	Blastocyst
0 mg/mL	191	154 (80.6)	85 (44.5)	65 (34.0)	48 (25.1)
0.6 mg/mL	199	135 (67.8)	86 (43.2)	69 (34.7)	56 (28.1)

Five replicates were performed.

The data was not statistically difference ($P > 0.05$).

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Table 9. Effects of L-carnitine addition to IVM, IVF and IVC medium on bovine blastocyst quality

Groups	N	Mean (± S.D.) number of cells in blastocyst			
		TE	ICM	Total cells	ICM ratio (%)
0 mg/mL	30	84.5 ± 9.9	23.9 ± 5.6	108.3 ± 10.6	22.0 ± 4.4
0.6 mg/mL	30	85.3 ± 10.6	23.1 ± 3.8	108.5 ± 11.3	21.4 ± 3.6

Five replicates were performed.

The data was not statistically difference ($P > 0.05$).

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CHAPTER V

OVERALL CONCLUSIONS

The advantage of IVP is the production of great numbers embryos at low price. On the other hand, the qualities of embryos *in vitro* are lower than those *in vivo* produced embryos. Due to oocytes matured *in vitro* have a reduced capacity to complete nuclear maturation and be fertilized compared to *in vivo* (Dunning et al., 2011). Development of a suitable culture system for IVM of oocyte is a major component of the IVP procedures (Gasparrini, 2002). Previous studies found that L-carnitine supplementation to IVM medium could enhance nuclear maturation and embryo development of porcine oocytes (Somfai et al., 2011; Wu et al., 2011). When L-carnitine was supplemented to culture medium, the blastocyst rates in mouse embryos were increased (Abdelrazik et al., 2009).

In the present study, supplementations of L-carnitine in IVM medium improved the nuclear maturation rates of bovine oocytes and their subsequent development to the blastocyst stage after IVF. The highest 8-cell, morula and blastocyst rates were obtained in 0.6 mg/mL of L-carnitine group compared with other groups. However, there was no significant difference in TE and ICM cell numbers among those groups. In addition, there was no positive effect of the addition of L-carnitine in IVM medium for bovine oocytes on their survival, cleavage and blastocyst rates after vitrification. Moreover, supplementation with L-carnitine throughout the entire IVP process (IVM, IVF and IVC) did not improve

developmental capacity and quality of resulting bovine embryos.

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BIOGRAPHY

Ms. Teewara Phongnimitr was born on July 25, 1983 in Saraburi, Thailand. She studied high school at Saraburiwitthayakhom School. She graduated with a bachelor degree of Science in Biochemistry from the Faculty of Science, Kasetsart University in Year 2006. After graduation, she worked as scientist and embryologist for 3 years in Fertility and Gynecology Endoscopy Center at Vibhavadi Hospital. At that time, she learnt the technique of assisted reproductive technology in human in the department of Obstetrics and Gynecology, Faculty of Medicine at Chulalongkorn University. Then, she decided to study Master degree course in School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology with Assoc. Prof. Dr. Rangsun Parnpai. She conducted the research in the topic of Effects of L-carnitine on maturation, cryo-tolerance and embryo developmental competence of bovine oocytes after *in vitro* fertilization as thesis work. The results from some part of this study have been presented as oral presentation at the 15th AAAP Animal Science Congress in Thailand, November 26-30, 2012.