Improvement of *in vitro* embryo production by controlling endoplasmic reticulum stress and autophagy in cattle

（小胞体ストレスおよびオートファジー制御による

ウシ体外胚生産系の改良に関する研究）

Hafiza Khatun

2020

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Dissertation submitted to the United Graduate School of Agricultural Sciences,
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Doctor of Philosophy

by

Hafiza Khatun

2020

Dedication

This work is dedicated to my beloved son, Huzaifa Islam, parents and teachers for their unfailing encouragement.

Declaration

I declare that this dissertation titled as **"Improvement of *in vitro* embryo production by controlling endoplasmic reticulum stress and autophagy in cattle"** presented for the degree of Doctor of Philosophy is an original report of my research work, and has not been submitted for any other degree or professional qualification. The collaborative contributions and references were clearly indicated and acknowledged.

The research work was done under the guidance of Dr. Ken-ichi Yamanaka, Saga University, Japan.

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List of Abbreviations

ANOVA	*Analysis of variance*
BSA	*Bovine serum albumin*
cDNA	*Complementary DNA*
COC	*Cumulus oocyte complex*
CR1aa	*Charles rosenkrans 1 with amino acid*
DAPI	*4',6-diamidino-2-phenylindole*
DCHFDA	*2,7-dihydrodichlorofluorescein diacetate*
DMSO	*Dimethyl sulfoxide*
DNA	*Deoxyribonucleic acid*
DPBS	*Dulbecco's phosphate buffered saline*
FBS	*Fetal bovine serum*
ER	*Endoplasmic Reticulum*
ERAD	*Endoplasmic Reticulum-associated degradation*
gDNA	*Genomic deoxyribonucleic acid*
GSH	*Reduced glutathione*
h	*Hour (s)*
HS	*Heat stress*

ICM	*Inner cell mass*
IVEP	*In vitro embryo production*
IVC	*In vitro culture*
IVF	*In vitro fertilization*
IVM	*In vitro maturation*
IVP	*In vitro production*
min	*Minute(s)*
mRNA	*Messenger Ribonucleic acid*
OPU	*Ovum Pick up*
PBS	*Phosphate-buffered saline*
PVA	*Polyvinyl alcohol*
PVP	*Polyvinyl pyrrolidone*
PVDF	*Polyvinylidene fluoride*
qPCR	*Quantitative polymerase chain reaction*
RT-PCR	*Real time-polymerase chain reaction*
RT	*Reverse transcription*
RNA	*Ribonucleic acid*
ROS	*Reactive oxygen species*

S	*Second (s)*
SEM	*Standard error of the mean*
SCNT	*Somatic cell nuclear transfer*
TCM-199	*Tissue culture medium 199*
TUNEL	*Terminal deoxynucleotidyl transferase*
TE	*Trophectoderm*
TM	*Tunicamycin*
TritonX-100	*Polyoxyethylene (10) Octylphenyl Ether*
TUDCA	*Tauroursodeoxycholic acid*
V	*Volume*
WK	*Week (wk)*
W	*Weight*

List of Gene/Protein Symbols

ATF4	*Activating transcription factor 4*
ATF6	*Activating transcription factor 6*
ATG5	*Autophagy related 5*
ATG7	*Autophagy related 7*
BAX	*Bcl-2–associated X protein*
BCL-2	*B-cell lymphoma 2*
BMP15	*Bone morphogenetic protein 15*
CDX2	*Caudal Type Homeobox 2*
CHOP	*C/EBP homologous protein*
COX2	*Cyclooxygenase-2*
eIF2α	*Eukaryotic initiation factor 2 alpha*
FST	*Follistatin*
GDF9	*Growth differentiation factor 9*
GLUT5	*Glucose transporter 5*
GRP78/BIP	*Glucose regulated protein 78kDa/Binding immunoglobulin protein*
H2A	*Histone H2A*

HSPA1A	*Heat shock 70 kDa protein 1*
IFN-tau	*Interferon-Tau*
IRE1	*Inositol-requiring enzyme 1*
JNK	*c-JUN N-terminal kinases*
LC3	*Microtubule-associated protein light chain 3*
MnSOD	*Manganese superoxide dismutase*
mTOR	*Mammalian target of rapamycin*
NANOG	*Homeobox protein NANOG*
PERK	*Pancreatic ER kinase (PKR)-like ER kinase*
PI3K	*Phosphoinositide 3-kinase*
PLAC8	*Placenta associated 8*
POU5F1	*POU Class 5 Homeobox 1*
RN18S1	*RNA, 18S ribosomal 1*
XBP1	*X-box binding protein 1*

Abstract

The aims of this study were to demonstrate the stress-associated developmental events and the effects of stress-modulating agents on in vitro development and embryo quality in cattle. First, to investigate whether the regulation of oxidative stress by supplementing sericin, a potential antioxidant during in vitro culture (IVC) can contribute to the production of bovine embryos with high viability in terms of thermotolerance, in vitro fertilized-embryos were cultured in IVC medium supplemented with 0.1 % sericin until Day 7, and then exposed to heat stress (HS) at 40.5°C for 6 h in the sericin-free medium. Following HS, subsequently embryos were cultured in sericin-free medium until Day 8. The results show that the expression of genes for HSPA1A and BAX in blastocysts obtained from culture without sericin significantly increased ($P < 0.05$) by HS (at 40.5°C for 6 h on Day 7) treatment compared to the non-HS control group; whereas blastocysts obtained from culture with sericin showed significantly decreased ($P < 0.05$) these gene expression to a level comparable to that in the non-HS control group. In addition, expression of IFN-tau, a maternal pregnancy recognition gene was increased in blastocysts produced by sericin culture. Moreover, TUNEL-positive cells number was significantly lower in blastocysts produced by sericin culture than in the HS control group. These findings indicate that sericin supplementation during IVC is useful for production of embryo with high tolerance in cattle.

Next, to examine the effects of endoplasmic reticulum (ER) stress during IVC on developmental kinetics and cryo-tolerance in embryos, tauroursodeoxycholic acid (TUDCA) and/ tunicamycin (TM), an ER stress inhibitor and inducer, respectively, were supplemented during IVC. As a result, treatment of TUDCA (10 μM) restored the

detrimental effects of TM-induced ER stress impairments in embryos development and quality. In addition, TUDCA (10 µM) significantly suppressed reactive oxygen species (ROS) generation, apoptosis and expression of ER stress markers (GRP78, ATF4, ATF6, IER1, sXBP1, CHOP and BAX); while it increased anti-apoptotic BCL2 gene and glutathione levels compared to the control. Moreover, ER stress inhibition via TUDCA (10 µM) enhances embryo cryo-tolerance after vitrification. Based on these findings, a follow-up study conducted to examine whether ER stress attenuation via TUDCA during IVM influences oocyte developmental competences. The results show that addition of TUDCA (100 µM) during IVM significantly decreased ROS, apoptosis and ER stress-induced protein/gene levels in matured COCs; thereby increases the maturation rate, and subsequent embryos development. Collectively, these findings suggest that controlling ER stress during IVM or IVC improves in vitro embryo development with high cryo-tolerance.

Finally, to explore whether the regulation of embryo autophagic activity during culture influences preimplantation embryo development and quality at the viewpoint of gene expression, rapamycin was used to induce, while wortmannin was used to suppress embryo autophagic activity during IVC. Surprisingly, autophagy is highly activated in embryos at 4-cell stage by rapamycin treatment (100 nM), as evidenced by significant upregulation of autophagy triggering molecules (LC3, ATG5, ATG7) and suppression of mTOR expression, and rapid maternal mRNA degradation. Further, induction of autophagy influences the blastocyst outcomes and activates many developmentally related genes (BCL2, MnSOD, SOX2, POU5F1, NANOG, PLAC8, IFN-tau, and GLUT5) in blastocysts. For the first, this research focus on the extent of embryo autophagic activity via rapamycin during culture involves with activation of many genes that are essential for blastocyst outcomes, quality and viability. In conclusion, these findings suggest that an appropriate balance between

oxidative and ER stress along with regulation of autophagy during culture influences in vitro early embryogenesis and may contribute to the development of strategies for the production of bovine blastocysts with high developmental competence.

Chapter 1

General Introduction

In vitro embryo production

Over the past 30 years, basic and applied studies on *in vitro* production (IVP) of embryos has generated a vast literature on factors regulating oocyte and embryo development, and quality. In addition, over this period, the IVP in cattle has showed impressive improvements and commercial bovine embryo transfer has become a large international business (Lonergan 2007). However, even though substantial progress has been made for improving the efficiency of *in vitro* production protocols in livestock, the rate of normally developing embryos remains lower than that of *in vivo*-derived embryos (Lonergan and Fair 2014). A variety stressor under *in vitro* conditions counteract the developmental competence of IVP embryos. Many studies have tried to improve the quality of IVP embryos by adding stress-reducing supplements with *in vitro* culture medium (Takahashi, et al. 2002). Nevertheless, the quality of IVP embryos is not comparable to that of *in vivo* embryos. Thus, the *in vitro* culture system requires further improvement by investing the stress-associated developmental events and the effects of stress-modulating agents on the early development and quality of embryos *in vitro.*

Oxidative stress

Oxidative stress is one critical factor inherent to IVP embryos; occurred by the accumulation of reactive oxygen species (ROS) that are generated within the cell as by-products of aerobic respiration and metabolism (Balaban, et al. 2005, Devine, et al. 2012). It is the result of an imbalance between the production and elimination of ROS in cells. ROS are highly reactive, ubiquitous and unstable molecules includes free radicals (hydroxyl ion, superoxide, etc.),

non-radicals (ozone, single oxygen, lipid peroxides, hydrogen peroxide) and oxygen derivatives. For becoming stable, ROS undergoes chain reactions to obtain electron from nucleic acids, proteins, carbohydrates and lipids; and thus, the attacked molecule loses its electron and becomes a free radical itself, beginning a chain reaction cascade which finally damages the living cell (Agarwal and Prabakaran 2005). Although normal level of ROS is beneficial for physiological processes such as tissue regeneration, hormonal signaling, intracellular redox regulation, and embryogenesis (Zhou, et al. 2013), however, excessive levels of ROS generate oxidative stress that has a negative effects on viability, gene expression, protein synthesis, development, and molecular signaling of gametes and embryos, all aspects that are related with of success of IVP embryos (Agarwal, et al. 2006, Devine, et al. 2012, Takahashi 2012). Indeed, ROS production is higher in embryos cultured *in vitro* compared to *in vivo* (Goto, et al. 1993). Mitochondria is the major site of ROS production and abundant ROS from mitochondria causes mitochondrial dysfunction (Fig. 1.1). Moreover, excessive ROS negatively impacts endoplasmic reticulum (ER) functions and triggers ER stress (Yoon, et al. 2014); behind ER stress itself can produce ROS (Landau, et al. 2013). This mechanistic link between ROS and ER stress is associated with mitochondrial dysfunction, which jeopardizes embryos developmental potential and leads cellular apoptosis (Landau, et al. 2013, Liu, et al. 2002, Somfai, et al. 2007). Recently, oxidative stress has been shown to be an initiator and major contributor to both ER stress and autophagy (Yuzefovych, et al. 2013), although the mechanisms underlying for these events are still unknown.

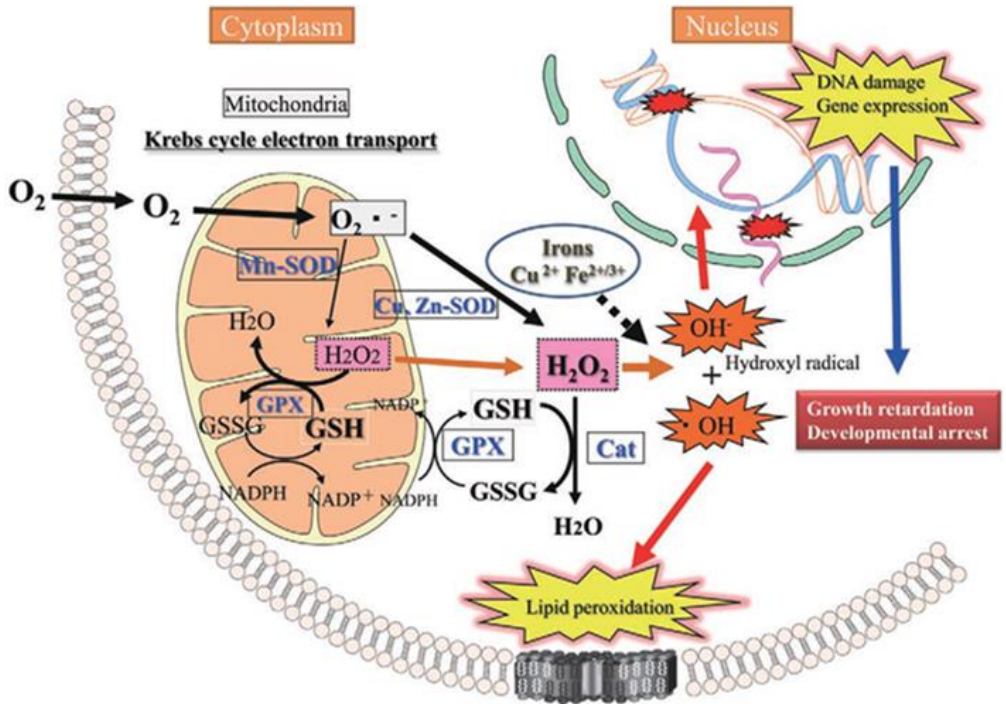

Figure 1.1 A schematic diagram of ROS generation and their effects on cellular functions (Takahashi 2012).

Endoplasmic reticulum (ER) stress

The ER is a major intracellular organelle that is found in the cytoplasm of eukaryotic cells and has multiple functions, particularly in the synthesis, folding, modification, and delivery of proteins to target sites via the secretory pathway (Yoshida 2007). The lumen of the ER is a unique environment, contains an array of chaperone systems such as glycosidases, Ca^{2+}-dependent chaperones and members of the protein disulfide isomerase (PDI) family which are responsible for the correct folding and transport of proteins. A balance between ER protein synthesis and folding machinery is essential for native protein folding. However, many phenomena, such as mutations, chemical treatments, or environmental stress can disrupt ER homeostasis, leading to the accumulation of misfolded and unfolded proteins, a condition known as ER stress, which causes cellular apoptosis, degeneration, and carcinogenesis (Ellgaard and Helenius 2003, Lindholm, et al. 2006, Tajiri, et al. 2004). When ER stress occurs, a pro-survival response known as the unfolded protein response (UPR) is activated to counteract ER stress and restore ER homeostasis (Lin, et al. 2019b).

The unfolded protein response signaling pathway

Three ER transmembrane protein sensors are involved in the unfolded protein response (UPR) pathway: a double-stranded, activated protein kinase-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1) (Kadowaki and Nishitoh 2013, Ron and Walter 2007). Under normal physiological conditions, the ER chaperone glucose-regulated protein, 78-kDa (GRP78), which is also known as binding immunoglobulin protein (BIP) binds with protein sensors and maintains the sensors as inactive; when misfolded proteins accumulate in the ER lumen, GRP78/BIP dissociates from the sensors and binds with misfolded proteins; resulting protein sensors are released and initiates a signaling cascade of the cytoprotective UPR (Fig. 1.2). In initial response of

UPR, PERK blocks general protein synthesis by phosphorylating eukaryotic initiation factor 2 (eIF2), which induces the expression of activating transcription factor 4 (ATF4) that upregulates the transcription of many genes essential for ER quality control. ATF6 translocate to the Golgi apparatus where it is cleaved to yield a transcription factor that initiates new chaperone synthesis to aid with folding of accumulated misfolded proteins. Phosphorylation of IRE1 inhibits protein synthesis and induces the splicing of x-box binding protein 1 (XBP-1), which converts it into an active transcription factor that regulates the transcription of ER chaperones to maintain cellular homeostasis by participating ER-associated degradation (ERAD) through the ubiquitin- proteasome system. The outcome of complex UPR signaling pathway depends on the severity and duration of the ER stress. The less severe stress can be overcome by promoting removal of unfolded protein to the cytoplasm for degradation and activation of autophagy to enable damaged cellular components to be eliminated (Kadowaki and Nishitoh 2013). However, if ER stress is excessive or prolonged, the UPR fails, and cellular apoptosis is induced by the activation of CCAAT-enhancer-binding-protein homologous protein (CHOP), Jun N-terminal kinase (JNK), and cleaved caspase 3 (Marre, et al. 2015, Yoshida 2007).

ER stress is a consequence of poor-quality oocytes/embryos

During oocyte maturation and early embryo development, various enzymes and metabolic pathways produce endogenous ROS; raised the cytoplasmic concentration of ROS level during maturation, alter the ER functions and induces ER stress (Burton, et al. 2017, Sato, et al. 2015).

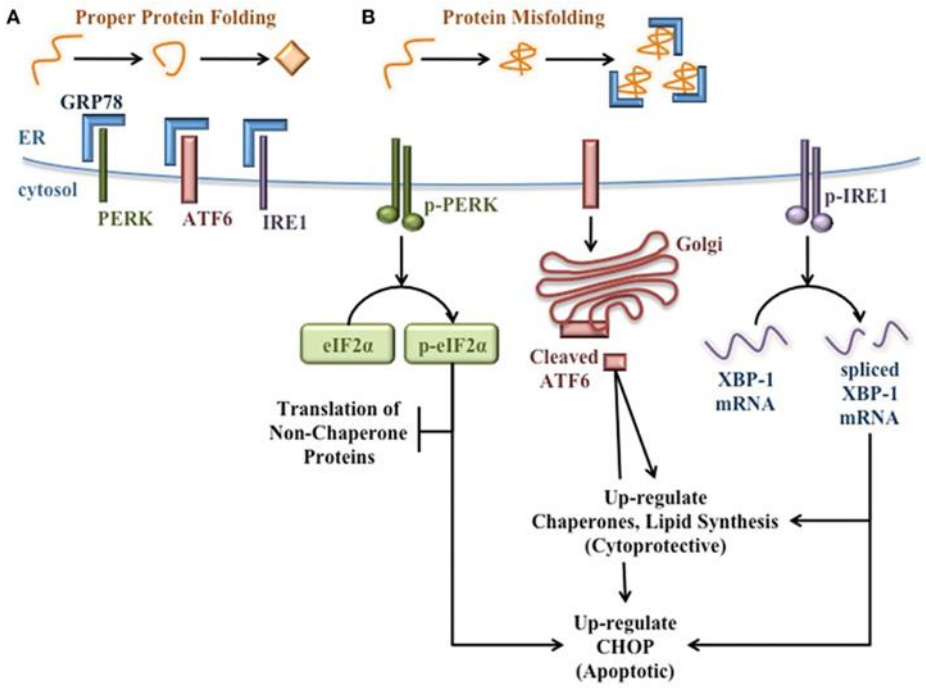

Figure 1.2 Signaling pathways of the unfolded protein response (Marre, et al. 2015). (A) When protein folding proceeds normally, the protein sensors of ER stress (PERK, ATF6, and IRE1) are bound and held in their inactive state by GRP78. (B) When misfolded proteins accumulate in the ER lumen, GRP78 binds misfolded proteins, thereby releasing the protein sensors of ER stress and allowing for the activation of the cytoprotective UPR. If ER stress is too great or prolonged, the UPR induces expression of pro-apoptotic proteins such as CHOP.

The ER is a major site for the biosynthesis of proteins, lipids and secretory proteins, and thus plays a key role in meeting the demand of new proteins for oocyte or early embryo development. Particularly, new protein synthesis due to the translation of maternal mRNA is extremely important for oocyte maturation and early embryo development (Guzel, et al. 2017). Indeed, homeostasis balance of ER is essential for normal developmental of oocytes/embryos. The classic ER stress marker genes/proteins, *ATF4*, *ATF6*, *XBP1*, *HSPA 5* and *GRP78*, have been detected in immature or mature oocytes of *in vitro* and *in vivo* derivations (Park, et al. 2018, Wu, et al. 2012, Yang, et al. 2012). These findings endorse that ER stress and UPR signaling are intrinsic in mammalian oocytes, and that their proper function could be essential for oocyte maturation and quality. Consequently, growing evidences reported that the ER stress-mediated activation of UPR signaling can be detected at the 2-cell to blastocyst stage and impairs i*n vitro* preimplantation and post-implantation embryo development (Basar, et al. 2014, Kim, et al. 2012, Lian, et al. 2011, Lin, et al. 2015). Furthermore, ER stress plays a negative role in developing somatic cell nuclear transfer (SCNT)-derived embryos obtained from electrofusion and drastically decrease the quality of SCNT embryos (Song, et al. 2011); appears that ER stress is stronger in electrofusion-derived SCNT embryos compared to *in vitro* fertilization (IVF)-derived embryos.

A beneficial approach to prevent ER stress-induced impairments in oocytes/embryos produced *in vitro* is the supplementation of ER stress inhibitor with *in vitro* maturation (IVM) and/ or *in vitro* culture (IVC) medium. The ER stress inhibitor not only improves oocyte maturation and preimplantation embryo development, it also prevents ER stress-mediated apoptosis by controlling ER stress-induced UPR signaling (Fig. 1.3).

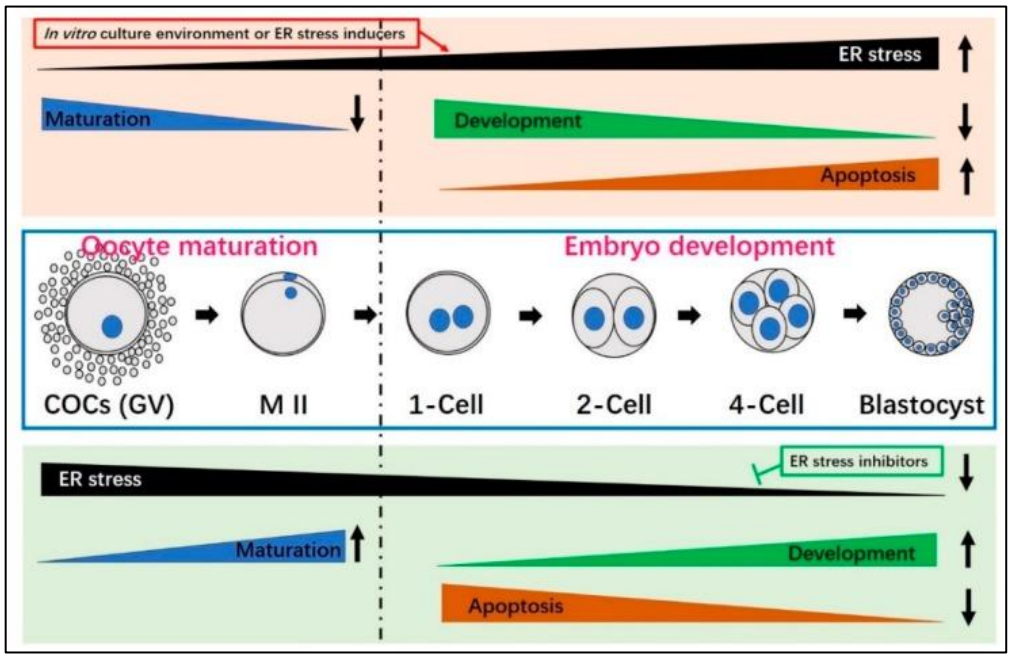

Figure 1.3 The influences of endoplasmic reticulum (ER) stress on oocyte maturation and preimplantation embryo development (Lin, et al. 2019b). Oocyte maturation and/or embryo culture environments associated with prolonged or severe ER stress (e.g., treatment with the ER stress inducer, TM) can significantly reduce oocyte maturation, decrease embryo developmental potential and increase apoptosis (top box with light pink color). When ER stress is inhibited by an ER stress inhibitor (e.g., TUDCA), maturation and development improve and the apoptotic index decreases (bottom box with light green color). Oocyte maturation is shown to the left of the broken black line, while embryo development is presented on the right. COCs, cumulus oocyte-complexes; GV, germinal vesicle; M II, metaphase II.

Tauroursodeoxycholic acid (TUDCA), is a chemical taurine conjugated form of ursodeoxycholic acid (UDCA), a unique bile acid that is mainly found in bears, with a trace amount in humans (Nunes, et al. 2012) and has been widely used to alleviate ER stress either *in vitro* or *in vivo* studies. Literature shows that TUDCA alleviates ER stress by mediating the ER-induced UPR signaling pathway and sustaining the ER homeostatic balance, consequently supporting oocyte maturation or early embryonic development (Basar, et al. 2014, Zhang, et al. 2012). TUDCA also known to improve the viability and subsequent embryo developmental potential of vitrified-warmed mice oocytes by reducing cryopreservation-induced ER stress (Zhao, et al. 2015). In addition to TUDCA, salubrinal, melatonin, valproic acid, or glutathione (GSH) supplementation also can reduce ER stress by regulating the UPR pathway. Thus, elucidation of new and relevant ER stress coping responses in preimplantation embryos might contribute to a comprehensive understanding of the regulation of normal embryonic development *in vitro.*

Autophagy

The term "Autophagy" (or autophagocytosis) derived from the Greek words "auto" meaning self and "phagy" meaning eating. Autophagy (self-eating) is an evolutionarily bulk degradation system in eukaryotes by which cytoplasmic double-membrane structures containing some organelle are delivered to the lysosome for degradation via the formation of autophagosomes (Mizushima 2018). Autophagy also an efficient recycling system or a pro-survival mechanism, as amino acids produced by autophagic degradation can be reused by the cells and protects cell from the aggregation of misfolded or damaged organelles. It can be triggered by both extracellular (nutrient deprivation, hypoxia, oxidative stress etc.) and intracellular ER stress, accumulation of damaged organelles, and aggregation of proteins etc.) stress conditions and plays a crucial role in physiological processes, like as

cell differentiation and development, stemness maintenance, prevention of cellular ageing, intracellular quality control, and clearance (Mizushima and Levine 2010, Yin, et al. 2016).

Modes of autophagy

Autophagy occurs through various mechanisms such as- macroautophagy, microautophagy, and chaperone-mediated autophagy (Wada, et al. 2014). Macroautophagy (simply referred as autophagy in this study) involves the formation of autophagosomes that subsequently fuse with the lysosome. It is the basic processes of protein degradation and recycling of the necessary cellular building blocks of glucose, amino acids, and fatty acids. In microautophagy, lysosomal compartments invaginate and engulf a portion of the cytosol along with organelles, forming membrane-bound spherical bodies within lysosomes. In chaperone-mediated autophagy, substrates are translocated across the lysosome membrane and delivered directly into the lumen (Fig. 1.4).

The progress of autophagy is regulated by autophagy related genes (Dunning, et al. 2007) including ATG8/LC3, ATG5, ATG6/BECLIN, ATG7 and their associated proteins (Mizushima, et al. 2010). The mammalian target of rapamycin complex 1 (mTORC1) is a negative regulator of autophagy; its inhibition also induces autophagy (Yamamoto, et al. 2014, Yang and Klionsky 2010). For a better understanding of the regulation of autophagy, chemical modulators of autophagy inhibitors or inducers such as wortmannin or rapamycin, respectively, are widely used by the researcher.

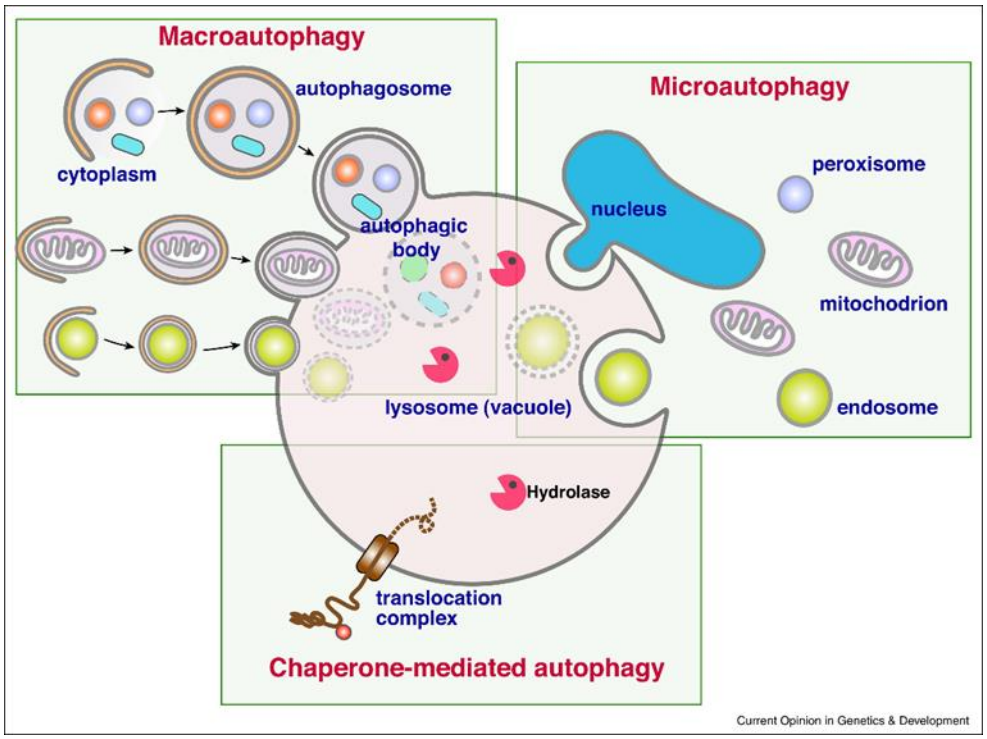

Figure 1.4 Modes of macroautophagy, microautophagy, and chaperone-mediated autophagy (Wada, et al. 2014).

Role of autophagy in embryogenesis

In the field of reproduction, the role of autophagy in oocytogenesis, embryogenesis, implantation, placentation, and delivery has become clearer. During the developmental progression from oocyte to cleavage stage embryo, maternal proteins and RNAs are degraded and quickly replaced by newly synthesized embryonic counterparts. The ubiquitin-proteasome and autophagy-lysosome systems are two major protein degradation pathways. The ubiquitin proteasome system involves in the removal of short-lived proteins; whereas long-lived proteins and organelles are degraded by autophagy (Tsukamoto, et al. 2008a). In addition to maternal macromolecules and proteins degradation, autophagy is essential to obtain a pool of free amino acids for protein synthesis, renovate cells or modify their external appearance. Autophagy-deficient mouse embryos die during preimplantation development (Tsukamoto, et al. 2008b); whereas, induction of autophagy augments the developmental competence of oocytes/embryos *in vitro* (Song, et al. 2012). A recent work reported that embryo autophagic activity during culture are positively correlated with embryo viability (Tsukamoto, et al. 2014). However, the mechanism by which autophagy influences the developmental competence and viability of embryos has not been fully evaluated yet. Thereby, it is important to investigate whether embryo autophagic activity during culture involves with transcriptional regulation or not; which may be one of the possible causes of increasing embryo viability rate.

In mammalian embryos, ER stress and autophagy serve as important developmental events, and those are highly influenced by the oxidative stress during culture. Despite, ER stress-induced dysfunction and role of autophagy has been well observed in mice, but it remains largely unknown in bovine embryogenesis. Therefore, this study has done to address the precise role and mechanisms of ER stress and autophagy in early bovine embryogenesis

under *in vitro* conditions. These findings reveal a novel mechanism governing preimplantation embryonic development *in vitro* and may contribute to the production of high-quality bovine embryos for successful fresh transfers or cryopreservation. The objectives of this study were-

1. To examine the effects of oxidative stress and redox regulation on preimplantation embryonic development and their quality in cattle.

2. To determine the relationship between ER stress and developmental kinetics of bovine embryos during IVC and its effect on embryo cryo-tolerance.

3. To investigate whether inhibition of ER stress during IVM enhances the developmental potential of bovine oocytes.

4. To explore whether induction of autophagy improves the developmental competence and quality of bovine embryos produced *in vitro.*

Chapter 2

Oxidative stress and redox regulation on preimplantation embryonic development and their quality in cattle

2.1 Abstract

Recently, the silk protein sericin has been identified as a potent antioxidant against oxidative stress and widely used in cell/embryo culture. Considering this strong potentiality of sericin against oxidative stress, the aim of this study was to examine whether sericin supplementation during IVC can contribute to the production of bovine embryos with high viability in terms of thermotolerance. For this purpose, IVF derived-embryos were cultured in IVC medium supplemented with 0.1 % sericin until Day 7, and then exposed to heat stress (HS) at 40.5°C for 6 h in the sericin-free medium. Following HS, subsequently embryos were culture in sericin-free medium until Day 8. The results show that the expression of genes for *HSPA1A* and *BAX* in blastocysts obtained from culture without sericin significantly increased ($P < 0.05$) by HS (at 40.5°C for 6 h on Day 7) treatment compared to the non-HS control; whereas blastocysts obtained from culture with sericin showed significantly decreased ($P < 0.05$) these gene expression to a level comparable to that in the non-HS control group. In addition, expression of *IFN-tau*, a maternal pregnancy recognition gene was increased in blastocysts produced by sericin culture. Moreover, TUNEL-positive cell numbers were significantly lower in blastocysts produced by sericin culture than in the HS control group. However, the rate of development to the blastocyst stage on Day 8 was not changed by HS treatment. These findings suggest that the supplementation of sericin with culture medium might be a useful technique to produce IVP embryo with high thermotolerance.

2.2 Introduction

Oxidative stress is considered one of the major causes of poor development of bovine embryos *in vitro*. It is a state in which the concentration of ROS increases above its biologically normal levels (Sikka 2001), resulting an imbalance of the intracellular redox potential toward an oxidized potential (Balaban, et al. 2005). The oxidative modification of cell components due to the action of ROS is one of the most potentially damaging processes for normal cell function, leading to inactivation of proteins, lipid membrane peroxidation, and DNA alterations (Yang, et al. 1998). ROS have been suggested to participate in meiotic arrest in oocytes and embryonic block and cell death (Hashimoto, et al. 2002, Nakamura, et al. 2002). The recent discovery that some transcription factors involved in diverse developmental processes are regulated by the intracellular redox potential (Dickinson, et al. 2002, Funato, et al. 2006, Zhang, et al. 2002), and these factors are very sensitive to oxidation by ROS or *S*-glutathionylation or require NAD(P)H (the reduced form) or $NAD(P)^+$ (the oxidized form). It seems that ROS production can be associated with structural events related to early embryo development and viability of blastocysts after transfer.

On the other hand, even though substantial progress has been made for improving the efficiency of *in vitro* production protocols in mammals, the pregnancy rates of IVP embryos still unsatisfactory (Ealy, et al. 2019). Coincidently, a recent study reported that frozen-thawed blastocysts, which have been widely used for embryo transfer, showed drastically decreased viability upon Heat stress (Mori, et al. 2015). Therefore, based on the assumption that embryo transfer would be performed to improve conception rates during the hot season, the production of IVP embryos with higher viability including thermotolerance would be also desirable.

Evidences suggest that the articulations of culture media by adding antioxidant/other regulatory molecules and reducing oxygen tension at the time of culture medium are the best ways to minimize oxidative stress and improves cell/embryo quality (Lee, et al. 2004, Takahashi 2012). Recently, the silk protein sericin has been identified as a potent antioxidant and widely used to reduce oxidative stress and maintain redox status during culture of mammalian cells/embryos. Sericin, a water-soluble globular protein (a protein hydrolysate) derived from silkworm Bombyx mori, surrounds two fibroin filaments in silk thread to form a cocoon and comprises 25%–30% of the cocoon weight (Craig and Riekel 2002). Due to its unique biological functions such as stimulation of cell proliferation and antioxidant, wound healing, anticancer, antibacterial, and moisturizing effects, sericin isolated by the degumming process during silk processing has been extensively used as a biomaterial in the pharmaceutical and cosmetic industries (Kunz, et al. 2016).

Sericin have strong polar side groups such as hydroxyl, carboxyl, and amino groups, which enable sericin to crosslink, copolymerize, and combine with other polymers (Dash, et al. 2009). Sericin is rich in serine and threonine (Cho, et al. 2003), which has a high hydroxyl group content. This unique amino acid composition provides sericin with antioxidant activity (Takahashi, et al. 2005). Addition of sericin with culture medium improves the developmental competence of bovine embryos cultured under oxidative stress (Isobe, et al. 2012). Moreover, evidence has been presented to support the successful use of sericin in cryopreservation of numerous mammalian cells/embryos (Isobe, et al. 2013, Miyamoto, et al. 2010). The replacement of fetal bovine serum (FBS) with sericin during IVM enlarged the perivitelline space, increased hyaluronic acid production, and decreased polyspermy fertilization in bovine oocytes (Hosoe, et al. 2014). Previously, we also observed that sericin has ani-heat stress properties, as evidence by markedly increasing quality of IVP bovine

embryos exposed to heat stress during culture. Together, these findings indicate that sericin has not only an antioxidative function but also variable biological functions, and sericin supplementation is thus expected to have multiple effects on IVP embryo. However, no studies to date have examined the effects of sericin on the culture of IVP embryo to improve embryo viability in terms of thermotolerance. Therefore, the objective of the present study was to estimate whether sericin supplementation in IVC medium can contribute to the production of bovine embryos with high viability from the viewpoint of thermotolerance.

2.3 Materials and methods

2.3.1 Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

2.3.2 Oocyte collection and *in vitro* maturation

Bovine ovaries were collected from a local abattoir and washed at least three times in sterile saline containing 100 IU/mL penicillin and 100 μg/mL streptomycin (Meiji Seika Pharma, Tokyo, Japan). Cumulus oocyte complexes (COCs) were aspirated from follicles (2–6 mm in diameter) using a 19-gauge needle attached to a 10-mL syringe and washed three times with TCM-199 (Cat. No. 12340030, Thermo Fisher Scientific, Waltham, MA, USA) containing 5% (v/v) FBS (Thermo Fisher Scientific). A group of 50 COCs were matured in a 4-well multidish (Cat. No. 176740, Nunc, Roskilde, Denmark) containing 500 μL TCM-199 supplemented with 5% (v/v) FBS, follicle-stimulating hormone (0.02 IU/mL; Kyoritsu Seiyaku, Tokyo, Japan), and gentamicin (10 μg/mL; Cat. No. 16672-04, Nacalai Tesque, Kyoto, Japan) covered with liquid paraffin (Cat. No. 26114-75, Nacalai Tesque) at 38.5 °C for 22 h in a humidified atmosphere of 5% CO_2 in air.

2.3.3 *In vitro* fertilization and embryo culture

For IVF, frozen semen was thawed by immersing the straw in warm water (37 °C) for 20 s. Spermatozoa were washed by centrifugation (800*g* for 10 min) in 90% (v/v) percoll solution (Cat. No. 17-0891-02, GE Healthcare, Chicago, IL, USA). After removing the supernatant, the pellet was diluted with IVF100 solution (Cat. No. IFP9630, Research Institute for the Functional Peptides, Yamagata, Japan) and centrifuged at 600*g* for 5 min. The spermatozoa

pellet was then diluted with IVF100 to prepare a final sperm-cell concentration of 5.0×10^6 sperm/mL. Following maturation, COCs were washed three times with IVF100 and IVF was performed at 38.5ºC in 5% CO_2 in air under humidified conditions for 6 h (25 oocytes per 100 μL sperm drop covered with liquid paraffin).

After IVF, cumulus cells were removed mechanically by pipetting in CR1aa medium (Rosenkrans, et al., 1993) containing 5% (v/v) FBS, and putative zygotes with polar bodies were placed into microdrops (20–25 zygotes per 50 μL drop) of CR1aa medium supplemented with 5% (v/v) FBS. The drops were then covered with liquid paraffin and cultured at 38.5 °C in a humidified atmosphere of 5% O_2 and 5% CO_2 and balanced with N_2 through Day 8 (Day 0 represented the day of insemination).

2.3.4 Sericin

Pure sericin (Cat. No. 167-22681, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was used in this study. Pure sericin was prepared by boiling silkworm (B. mori) cocoon shells under alkaline conditions and then drying the extract to a powder. The average molecular weight of sericin was approximately 33 kDa. The amino acid composition of the sericin was Ser, 34.2; Asp, 16.4; Gly, 14.8; Thr, 8.2; Arg, 6.1; Ala, 4.6; Glu, 3.8; Val, 3.6; Tyr, 3.1; Lys, 1.9; His, 1.8; Leu, 1.0; I-leu, 0.5; Pro and Ph-ala, <0.05 (mol %).

2.3.5 Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)

To evaluate the presence of apoptotic cells in blastocysts, a TUNEL assay kit (in situ cell death detection kit; Roche, Basal, Switzerland) was used according to the manufacturer's guidelines. In brief, blastocysts were fixed in 4% (w/v) paraformaldehyde solution (pH 7.4)

for 40 min, rinsed three times in PBS containing 0.05% (w/v) PVP (Cat. No. 28354-75, Nacalai Tesque; PVP-PBS), and then permeabilized in PVP-PBS containing 0.5% TritonX-100 (Cat. No. 169-21105, FUJIFILM Wako Pure Chemical Corporation) for 20 min, followed by three washes with PVP-PBS for 5 min. The fragmented DNA ends of the cells were labeled with fluorescein-dUTP for 60 min at 38.5 °C. After incubation, blastocysts were washed three times in PVP-PBS for 5 min each, followed by mounting onto glass slides using mounting solution containing 4′,6-diamidino-2-phenylindole (DAPI; Vectashield with DAPI; Vector Laboratories, Burlingame, CA, USA). The fluorescence of the fragmented DNA ends was detected using a fluorescence microscope (EVOS® FL, Thermo Fisher Scientific).

2.3.6 cDNA synthesis and quantitative polymerase chain reaction (qPCR)

Total RNA from 5 to 10 blastocysts was isolated using an RNeasy Micro Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. Extracted RNA (about 12 μl) was either used immediately for reverse transcription (RT) or stored at −80 °C until use. The RT was performed using the SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The RT mix (20 μl) consisted of 12 μl of RNA, 4 μl of reaction mix, 2 μl of enzyme mix, and 2 μl of nuclease-free water. The RT reaction was performed with annealing of random primers (10 min at 25°C), cDNA synthesis (60 min at 42°C), and termination of the reaction (5 min at 85°C). Each sample was analyzed in duplicate using the SsoFast™ Evagreen® Supermix with CFX Connect (Bio-Rad, Hercules, CA, USA). The real-time PCR mix (20 μl) consisted of 2 μl of cDNA, 10 μl of supermix, 6 μl of nucleasefree water, and 1 μl each of forward and reverse primers (10 pmol, Table 2.1) for each gene. The program was used for the amplification included an initiation cycle (30 s at 95°C), 40 cycles of PCR (95°C for 5 s and 58°C for 5 s), and a

melting curve program (60–95°C with a heating rate of 0.5°C/s, continuous fluorescence acquisition, and cooling to 30°C). The fold changes of target genes were evaluated by the ΔΔCT method using endogenous reference gene (*RN18S1*) expression. The quality of the PCR products obtained was validated using melting curve analysis.

2.3.7 Experimental design

To confirm whether antioxidant sericin supplementation during IVC enhances the thermotolerance capability of blastocyst, embryos were cultured in IVC medium with or without 0.1% (w/v) sericin until Day 7, after which they were washed three times with new IVC medium without sericin followed by exposed to heat stress (HS) at 40.5°C for 6 h in sericin-free IVC medium. Immediately following HS treatment, some blastocysts were subjected to examine the expression of many developmentally related genes involved in antioxidant protection (*MnSOD*), stress-response (*HSPA1A*), apoptosis (*BAX*), pluripotency *(POU5F1*), and implantation/pregnancy (*CDX2*, *COX2*, *IFN-tau*, and *PLAC8*) by using qPCR; and remaining embryos were subsequently cultured without sericin until Day 8. The rate of development to blastocysts and the percentage of TUNEL-positive cells in blastocysts were evaluated on Day 8. For both experiments, as a control, embryos were cultured in IVC medium without sericin at 38.5°C until analysis.

2.3.8 Statistical analysis

All data were obtained from six replicates. The developmental rates were expressed as the mean. The other data were expressed as the mean ± standard error of the mean (SEM). *In vitro* development data were analyzed using chi-squared tests. Other data were analyzed using analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison

tests. All percentage data were arcsine-transformed before statistical analysis. $P < 0.05$ denoted a statistically significant difference.

Table 2.1 List of primer information's

Gene	Primer sequence	Product size (bp)	Accession no.
RN18S1	F: aaacggctaccacatccaagg R: gcggaaggatttaaagtggactc	138	DQ066896
HSPA1A	F: gacaagtgccaggaggtgattt R: cagtctgctgatgatggggtta	117	U09861
MnSOD	F: gcttacagattgctgcttgt R: aaggtaataagcatgctccc	101	S67818.1
IFN-tau	F: tccatgagatgctccagcagt R: tgttggagcccagtgcaga	103	X65539
POU5F1	F: tgcagcaaattagccacatc R: aatcctcacgttgggagttg	123	NM_174580.2
BAX	F: ctactttgccagcaaactgg R: tcccaaagtaggagagga	158	NM_173894.1
PLAC8	F: cggtgttccagaggtttttcc R: aagatgccagtctgccagtca	163	NM_016619
CDX2	F: gccaccatgtacgtgagctac R: acatggtatccgccgtagtc	140	XM_871005
COX2	F: aagcctagcactttcggtggagaa R: tccagagtgggaagagcttgcatt	133	DQ066897

2.4 Results

2.4.1 Gene expression analysis

As shown in Fig. 2.1A and B, blastocysts obtained by culture without sericin when exposed to HS (at 40.5°C for 6 h) on Day 7 showed significantly higher ($P < 0.05$) expression of *BAX* and *HSPA1A* genes compared with that in the non-HS control group. On the other hand, blastocysts obtained by culture with sericin when exposed to HS (at 40.5°C for 6 h) on Day 7 showed the same expression levels of these genes in as that in the non-HS control group. Moreover, the expression of *IFN-tau* was also significantly increased ($P < 0.05$) in blastocysts obtained by culture with sericin compared with that in the other conditions (Fig. 2.1C). However, the expression of *POU5F1*, *PLAC8*, *MnSOD*, *CDX2*, and *COX2* in blastocysts did not significantly differ among groups (Fig. 2.1D–H).

2.4.2 Developmental rate and apoptosis status

The rate of development to the blastocyst stage on Day 8 was not changed by HS treatment (Fig. 2.2A). However, the percentage of TUNEL-positive cells in blastocysts obtained from culture without sericin significantly increased ($P < 0.05$) by HS (at 40.5°C for 6 h on Day 7) treatment; whereas blastocysts obtained from culture with sericin significantly decreased ($P < 0.05$) the percentage of TUNEL-positive cells to a level comparable to that in the non-HS control group (Fig. 2.2B).

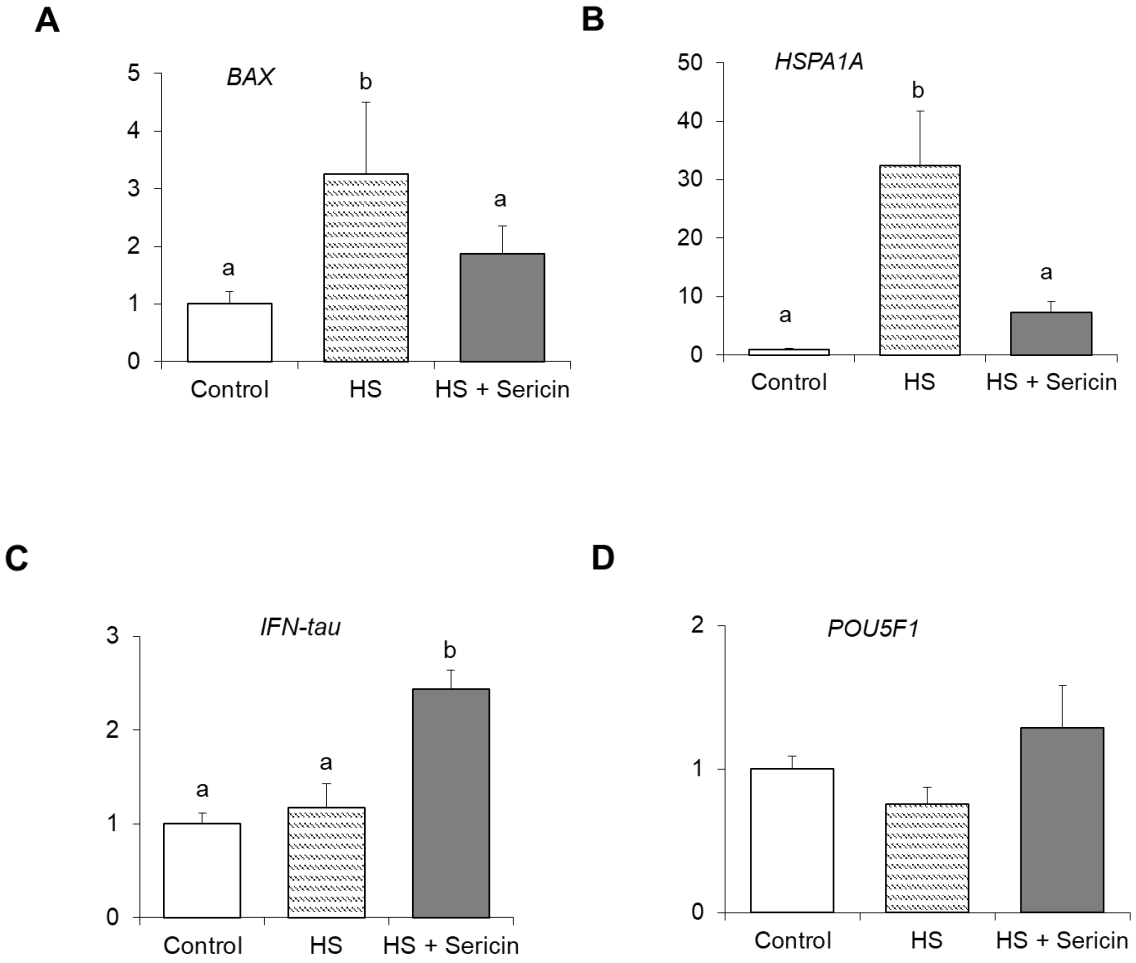

Figure 2.1 Gene expression analysis in blastocysts after heat stress. Embryos were cultured in IVC medium with (HS + Sericin) or without (Control and HS) sericin until Day 7. Blastocysts from each group were transferred to a drop of new IVC medium without sericin, and those in the HS and HS + Sericin groups were exposed to heat stress at 40.5°C for 6 h on Day 7 followed by qPCR analysis. Different letters indicate a significant difference ($P < 0.05$). HS: heat stress, IVC: *in vitro* culture, qPCR: quantitative polymerase chain reaction.

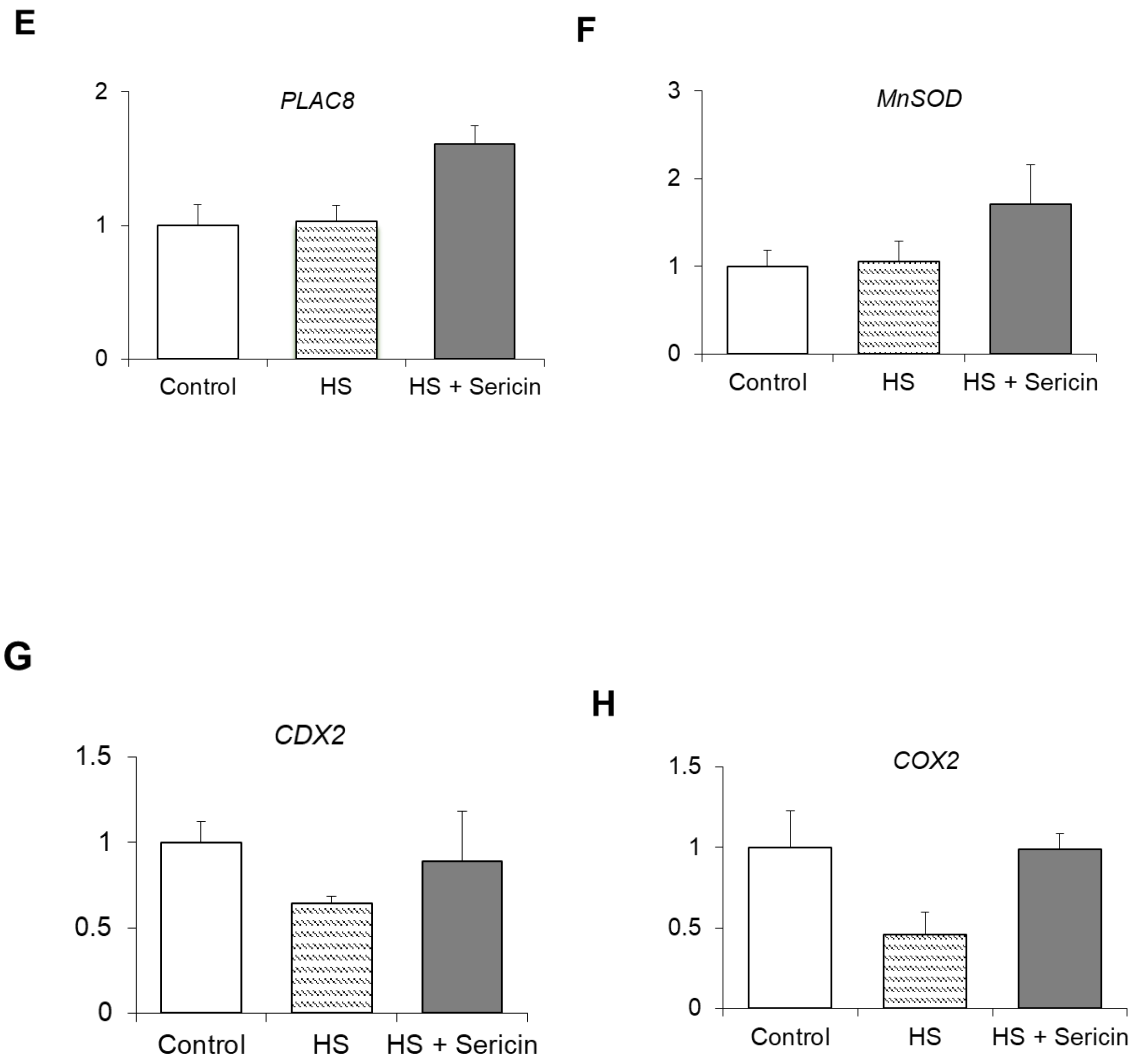

Figure 2.1 Continuation…….

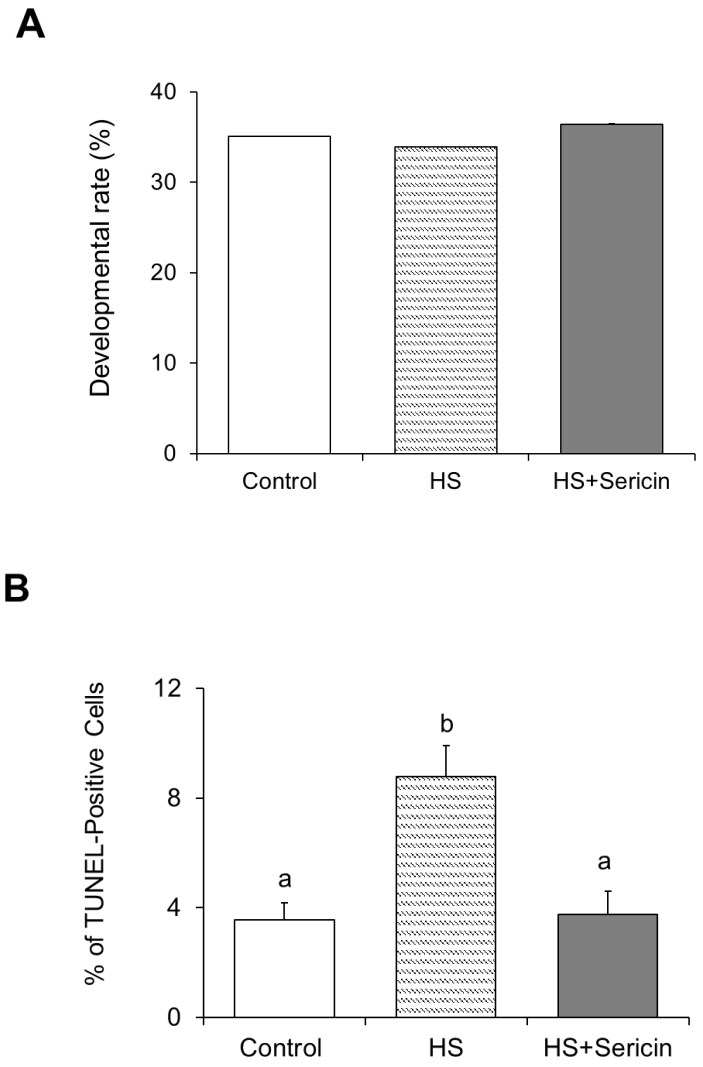

Figure 2.2 Thermotolerance of blastocysts produced in sericin supplemented medium. Embryos were cultured in IVC medium with (HS + Sericin) or without (Control and HS) sericin until Day 7, after which they were washed three times with new IVC medium without sericin. After washing, embryos in HS and HS + Sericin groups were exposed to heat stress at 40.5°C for 6 h. Embryos were subsequently culture at 38.5°C until Day 8 following Heat stress treatment. The blastocyst rate (A) and percentage of TUNEL-positive cells (B) were evaluated on Day 8. In a total, 402 embryos were cultured in six replicates, and 40 blastocysts were subjected to TUNEL assay. Values are the mean ± SEM, and different letters denote significant differences ($P < 0.05$). HS: heat stress; IVC: *in vitro* culture; TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

2.5 Discussion

The present study demonstrates for the first time that sericin supplementation could contribute to the production of bovine embryos with high viability in terms of thermotolerance. The results show that quality was maintained in blastocysts obtained from the culture with sericin, as evidenced by the lack of change in the apoptosis index even upon removal of sericin from the culture medium during HS; indicating that embryos obtained from the culture with sericin possess high thermotolerance.

Moreover, gene expression analysis also supported the high thermotolerance of blastocysts obtained from the culture with sericin. The mRNA expression of *BAX*, a pro-apoptotic protein that induces apoptosis through release of cytochrome c from mitochondria to the cytoplasm (Gu, et al. 2015), was significantly increased by HS, which might underlie the higher incidence of apoptosis. In contrast, *BAX* expression was significantly reduced in blastocysts derived from the culture with sericin. Accordingly, the mRNA expression of *HSPA1A* in blastocysts derived from the culture with sericin was significantly decreased compared with that in blastocysts derived from the culture without sericin, although *HSPA1A* expression was more than seven times higher than that in the non-HS control. *HSPA1A*, a marker of cell stress, assists refolding of misfolded and aggregated proteins as a molecular chaperone (Bukau 2005) and is upregulated by HS in bovine embryos (Mori, et al. 2015). This result indicates that sericin likely relieves protein denaturation caused by HS. Alternatively, *IFN-tau* is produced by trophoblasts and is known as a maternal pregnancy recognition gene. In this study, *IFN-tau* expression was significantly higher in blastocysts obtained from the culture with sericin than in those from other culture conditions. Nevertheless, the results of the gene expression analysis support the high thermotolerance of blastocysts obtained from the culture with sericin. Therefore, the present study provides

a scientific basis for new IVC medium formulation with sericin and may provide a useful approach for producing high-quality IVP bovine embryos under commercial setting.

Chapter 3

Relationship between endoplasmic reticulum stress and developmental kinetics of bovine embryos during *in vitro* culture and its effect on embryo cryo-tolerance

3.1 Abstract

ER stress, a dysfunction in protein folding capacity of the ER, is involved in many physiological responses including mammalian reproductive systems. Many studies have shown that ER stress interferes with the developmental process of *in vitro* oocyte maturation and embryo development; however, little is known about its effect on bovine preimplantation embryonic development. Therefore, the aim of this study was to examine the effects of ER stress during IVC on developmental competency and cryo-tolerance in bovine embryos. IVF-derived zygotes were cultured in CR1aa medium supplemented with TUDCA and/or TM, which are ER stress-inhibitory and stress-inducing agents, respectively, for 8 days. TM treatment decreased the blastocyst developmental rate and increased the percentage of apoptotic cells compared to that in the control group (10.2 ± 2.3% vs. 39.75 ± 1.3% and 17.8 ± 1.2% vs. 3.6 ± 1.1%, respectively; $P < 0.01$). However, the blastocyst developmental rate was increased, and the percentage of apoptotic cells was decreased by addition of TUDCA in IVC medium compared to that in the control group (50.9 ± 0.9% vs. 39.75 ± 1.3% and 1.13 ± 1.0% vs. 3.6 ± 1.1%, respectively; $P < 0.01$). Importantly, in the group treated with TM plus TUDCA, the developmental rate and the percentage of apoptotic cells in blastocysts were similar to that in the control group, indicating that TUDCA ameliorates the adverse effects of TM alone on embryo development and apoptosis. In addition, TUDCA treatment significantly reduced the ROS, expression of ER stress (*GRP78*,

ATF4, *ATF6*, *IER1*, and *sXBP1*) and pro-apoptotic (*CHOP* and *BAX*) genes, while it increased anti-apoptotic *BCL2* gene expression and glutathione levels. Moreover, TUDCA improved blastocyst cryo-tolerance as marked by a significantly increased hatching rate and decreased the number of apoptotic cells recorded at 48 h after a post-warming. These results suggest that modulation of ER stress during IVC contributes to the production of high-quality bovine embryos in terms of cryo-tolerance.

3.2 Introduction

Recent molecular studies have shed light on the impact of ER stress mediated UPR signaling on the mammalian reproductive system, including placental development, preimplantation embryonic development, implantation, and decidualization. Evidences show that ER stress is involved in ovarian granulosa cell apoptosis, meiotic maturation, and preimplantation embryonic development *in vitro* (Lin, et al. 2012, Wu, et al. 2013, Zhang, et al. 2012). Neither temporary nor permanent induction of ER stress by TM, an ER stress inducer appears to upstream the UPR signaling molecules that mediates ER stress–induced embryonic apoptosis (Basar, et al. 2014). Beyond the activation of ER stress in oocytes/embryos involves in epigenetic changes such as changes in histone acetylation, histone methylation and DNA methylation (Latham 2016). Additional changes in the activation states of transcription factors also occur, which may lead secondarily to changes in embryonic genome programing. Notably, treatment with ER stress inhibitors, TUDCA or salubrinal can promote oocyte/embryo survival and quality (Lin, et al. 2019b).

On the other hand, embryo vitrification is an indispensable approach for assisted reproductive technology; even extensive research on the method, impact of ER stress on the cryo-tolerance of *in vitro*-generated embryos remains unclear. It was known that ER stress response pathway is initiated at all stages of development, particularly at the eight-cell, morula, and blastocyst stages, but not during embryo vitrification (Abraham, et al. 2012). Therefore, the underlying hypothesis of this study is that ER stress is a recurrent phenomenon during IVC because of the high demand for protein synthesis and folding during blastocyst development, maintenance of ER homeostasis by UPR is vital for normal development of the preimplantation embryo and that excess UPR as a result of unfolded/misfolded protein overload induces mitochondrial dysfunction, blocks *in vitro*

blastocyst. However, attenuating ER stress via TUDCA during culture would improve preimplantation embryonic development with high cryo-tolerance.

Therefore, the present study aimed 1) to examine the effects of TUDCA on the developmental competence and quality of embryos produced *in vitro*, and 2) to investigate whether the inhibition of ER stress during IVC contributes to the production of embryos with enhanced cryo-tolerance.

3.3 Materials and methods

3.3.1 *In vitro* embryo production

Oocyte collection, IVM, IVF, and IVC were carried out according to procedures described in chapter 2 (section 2.3.2 and 2.3.3).

3.3.2 Chemical treatment

To investigate the effects of ER stress, embryos were cultured in IVC medium supplemented with TUDCA (Cat. No. 580549, Calbiochem, San Diego, CA, USA) or TM (Cat. No. 11445, Cayman Chemical Company, Ann Arbor, MI, USA), ER stress- inhibitory and -inducing agents, respectively. The chemicals were provided as a crystalline solid (TM), and a lyophilized powder (TUDCA) dissolved in dimethyl sulfoxide and distilled water, respectively, to give stock solutions. The stock solution TM (1 mg/mL) was stored at -20 °C until the day of use, and TUDCA (50 mM) was stored at 4 °C and used within 2 wk. The stock solutions were diluted in a CR1aa culture medium to make 10-, 50-, and 100-μM solutions of TUDCA and 1-μg/mL solution of TM. The TM concentration was based on data from a previous study (Lin, et al. 2016).

3.3.3 Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)

TUNEL was conducted based on the methods described in chapter 2 (see section 2.3.5).

3.3.4 Detection of intracellular ROS and reduced glutathione (GSH) levels

Intracellular ROS and GSH levels were measured on Days 2 and 7 of embryos by using fluorescence probes according to the manufacturer's guidelines. The fluorescent dye 2′,7′-

dichlorodihydrofluorescein diacetate (DCHFDA; Molecular Probes, Eugene, OR, USA) was used to detect intracellular ROS as green fluorescence and Cell Tracker™ Blue (Life Technologies, Carlsbad, CA, USA) was used to detect GSH level as blue fluorescence. At least 10 embryos from each treatment group were incubated in PVP-PBS containing 10 μM DCHFDA or Cell Tracker™ Blue for 15 min at 38.5 °C in a humidified atmosphere at 5% CO_2 in air, followed by two washes with PVP-PBS. The embryos were then placed on a glass bottom dish and green fluorescence emission was detected using a fluorescence microscope (EVOS® FL, Thermo Fisher Scientific). The fluorescence intensity of images was quantified using ImageJ software (version 1.55; National Institutes of Health, Bethesda, MD, USA) by counting the number of pixels after color inversion.

3.3.5 RT-PCR and qPCR analysis

Total RNA from blastocysts (approximately 10) was isolated using an RNeasy Micro Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Extracted RNA (approximately 12 μL) was either used immediately for RT or stored at - 80 °C until use. The RT was performed using a SuperScript™ IV VILO™ Master Mix cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The RT mix (20 μL) consisted of 12 μL of RNA, 4 μL of SuperScript™ IV VILO™ Master Mix, and 4 μL of nuclease-free water. The RT reaction was performed with the annealing of random primers (10 min at 25 °C), cDNA synthesis (10 min at 50 °C), and termination (5 min at 85 °C). Each sample was analyzed in duplicate using SsoAdvanced™ Universal SYBR® Green Supermix with CFX Connect™ (Bio-Rad, Hercules, CA, USA). The real-time PCR mix (20 μL) consisted of 2 μL of cDNA, 10 μL of supermix, 6 μL of nuclease-free water, and 1 μL each of forward and reverse primers for each gene. The program used for the amplification included a denaturing cycle (30 s at 95 °C), 40 cycles of PCR (95 °C for 5 s

and 58 °C for 5 s), and a melting curve program (60–95 °C with a heating rate of 0.5 °C/s, continuous fluorescence acquisition, and cooling to 30 °C). The fold-changes of target genes were evaluated by the ΔΔCT method using endogenous reference gene (*RN18S1*) expression. To measure spliced (s) and unspliced (u) *XBP1* gene expression, 2 μL of cDNA was used as a template for RT-PCR amplification in a 25-μL-reaction volume using GoTaq® Hot Start Green Master Mix Polymerase (Promega Madison, WI, USA) according to the manufacturer's instructions. The cycling conditions were 94 °C for 2 min, followed by 40 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 5 min. Gel electrophoresis (25 μL volume per sample) was performed on 4% agarose gel containing ethidium bromide (0.5 μg/mL) and visualized under ultraviolet light. Bands were quantified using ImageJ software (version 1.55; National Institutes of Health) and the intensity of each band was normalized to its corresponding reference gene *H2A* band to compare sample values quantitatively. The PCR primers used to amplify each gene are listed in Table 3.1.

3.3.6 Embryo vitrification

All vitrification procedures were performed at room temperature, and only expanded blastocysts at Days 7 and 8 were subjected to vitrification. Briefly, blastocysts were first washed in a basic vitrification (BV) medium which consisted of PBS + 20% FBS. Next, the blastocysts were placed in vitrification solution 1 (BV medium with 7.5% ethylene glycol (EG) + 7.5% DMSO) for 3 min and then in vitrification solution 2 (BV medium with 16.5% EG + 16.5% DMSO + 0.5 M sucrose) for 45–60 s. The blastocysts were then placed in a microdrop on the tip of the cryotop® (Kitazato Corporation, Shizuoka, Japan) and quickly transferred to liquid nitrogen. For warming, all procedures were performed on a warm plate (42 °C). After removing the cap, blastocysts on the cryotop were immediately exposed to a

warming solution (BV medium with 0.3 M sucrose) and washed in the warming solution (drop-by-drop) for 5 min. Finally, the blastocysts were washed in IVC medium and cultured for 2 days at 38.5 °C in an atmosphere of 5% CO_2, 5% O_2 and balanced with N_2. After 24 and 48 h post-warming, blastocyst survival was evaluated according to both morphological appearance and re-expansion of the blastocoel. Thereafter, the re-expansion and hatching rates as well as the apoptotic status of blastocysts were recorded.

3.3.7 Experimental designs

Experiment 1: Effects of TUDCA supplementation during IVC on developmental competence of bovine embryos

To assess the dose-dependent effects of TUDCA on preimplantation embryonic development, IVF-derived bovine embryos were cultured in CR1aa medium supplemented with different concentrations (0, 10, 50, and 100 µM) of TUDCA at 38.5 °C in a humidified atmosphere of 5% O_2, 5% CO_2, and balanced with N_2 to Day 8 (Day 0 represented the day of insemination). Developmental competence was determined from the cleavage and blastocyst rates on Days 2 and 8, respectively.

Experiment 2: Effects of TUDCA and/or TM on embryo development and quality

To investigate the effects of ER stress and efficacy of TUDCA supplementation during IVC, embryos were cultured in 4 different groups: (a) control; (b) TUDCA (10 µM); (c) TM (1 µg/mL); and (d) TUDCA + TM until Day 8 and developmental competence was examined as the cleavage and blastocyst rates on Days 2 and 8, respectively. The total cell number and apoptotic status of the blastocysts at Day 8 were also assessed to investigate embryo quality by performing a TUNEL assay.

Experiment 3: Effects of TUDCA on intracellular ROS and GSH levels in embryos

To determine the potential antioxidant effects of TUDCA on blastocyst formation and quality, embryos were treated either with or without TUDCA (10 µM) during IVC, and analyzed the intracellular ROS and GSH levels in embryos both on Days 2 and 7 by using DCHFDA and Cell Tracker™ Blue fluorescence, respectively.

Experiment 4: Effects of TUDCA on gene expression in embryos.

To examine the mechanism by which TUDCA counteracts the detrimental effects of ER stress, blastocysts at Day 8 were analyzed for expression of ER stress (*GRP78/BIP*, *IER1*, *CHOP*, *ATF4*, and *ATF6*) and apoptosis-related (*BAX* and *BCL2*) genes by qPCR. In addition, the *sXBP1/u*XBP1 expression ratio was measured by gel-based RT-PCR.

Experiment 5: Effects of TUDCA during IVC on development and quality of vitrified post warmed blastocysts

Embryos were treated either with or without TUDCA (10 µM) during IVC and only expanded blastocysts from Day 8 were subjected to vitrification. After 24 and 48 h post-warming, examined the re-expansion and hatching rates of blastocysts. Moreover, after 48 h post-warming, the total cell number and apoptotic status of vitrified blastocysts were assessed to investigate embryo quality in a TUNEL assay.

3.3.8 Statistical analysis

Each experiment was repeated at least five times. The results are presented as the mean ± SEM. All data were analyzed by ANOVA followed by the Tukey-Kramer multiple comparison test. All percentage data were arcsine-transformed prior to statistical analysis. A $P < 0.05$ was considered as statistically significant.

Table 3.1 List of primer used in PCR

Gene	Primer sequence	Product size (bp)	Accession number
BAX	F: agcgcatcggagatgaattg R: aaacatttcagccgccactc	110	NM_173894.1
GRP78/BIP	F: tgcagcaggacatcaagttc R: tttgtttgcccacctccaac	91	NM_001075148.1
XBP1	F: agaccaaggggaatggagcg R: ggggaggccggtaaggaact	288	NM_001034727.3
BCL2	F: tggatgaccgagtacctgaa R: gagacagccaggagaaatcaaa	123	NM_001166486.1
ATF6	F: tgaacttcgaggatgggttc R: gaatttgagccctgttccag	117	NM_001075935
ATF4	F: ggccaagcacttcaaacatc R: aagcatcctccttgctgttg	110	NM_001034342.2
CHOP	F: gcaccaagcatgaacagttg R: atcgatggtggttgggtatg	116	NM_001078163.1
IRE1	F: ccgaagttcagatggcattc R: tctgcaaaggctgatgacag	108	XM_001789477.1
RN18S1	F: aaacggctaccacatccaagg R: gcggaaggatttaaagtggactc	138	DQ066896
H2A	F: aggacgactagccatggacgtgtg R: ccaccaccagcaattgtagccttg	208	NM_174809

3.4 Results

3.4.1 TUDCA improves developmental competence of bovine embryos

In the first experiment, the optimal concentration of TUDCA for bovine embryo development *in vitro* was determined. The results show that addition of 10 μM TUDCA to the IVC medium improved the blastocyst formation rate compared to that observed in the control group (49.7 ± 2.5% vs. 38.7 ± 2.0%; $P < 0.01$; Table 3.2). Notably, the percentage of blastocysts did not significantly differ between the groups treated with 10 and 50 μM TUDCA, whereas the percentage was decreased ($P < 0.01$) by the maximum concentration (100 μM) of TUDCA (Table 3.2). However, no significant difference was found in the embryo cleavage rate among the groups, except for in the 100 μM TUDCA group. Therefore, all subsequent experiments were performed using 10 μM TUDCA.

3.4.2 TUDCA reverses the developmental defects induced by TM

As shown in Figures 3.1 and 3.2, TM treatment decreased the blastocyst developmental rate and increased the percentage of apoptotic cells compared to that in the control group (10.2 ± 2.3% vs. 39.8 ± 1.3% and 17.8 ± 1.2% vs. 3.6 ± 1.1%, respectively; $P < 0.01$). However, the blastocyst rate was increased and decreased the percentage of apoptotic cells by addition of TUDCA to the IVC medium compared to that in the control group (50.9 ± 0.9% vs. 39.8 ± 1.3% and 1.1 ± 1.0% vs. 3.6 ± 1.1%, respectively; $P < 0.01$). Importantly, in the group treated with TM plus TUDCA, the developmental rate and percentage of apoptotic cells in blastocysts were similar to those in the control group, indicating that TUDCA ameliorates the adverse effects of TM alone on embryo development. In contrast, no significant difference was found in the total cell number and embryo cleavage rate among the groups, except for the TM group.

Table 3.2 Effects of TUDCA supplementation during IVC on bovine embryonic development

TUDCA (μM)	No. of embryos cultured	No. of embryos cleaved (%)	No. of blastocysts (%)
0 (Control)	142	126 (89.4 ± 1.6)[a]	49 (38.7 ± 2.0)[a]
10	147	132 (89.8 ± 1.3)[a]	73 (49.7 ± 2.5)[b]
50	145	130 (89.7 ± 0.9)[a]	65 (44.8 ± 3.3)[ab]
100	142	108 (76.1 ± 3.4)[b]	32 (22.5 ± 2.6)[c]

The percent of embryos that cleaved was assessed on Day 2 and the percent of embryos that developed to the blastocyst stage was determined at Day 8 (day 0 represents the day of insemination). TUDCA: tauroursodeoxycholic acid; IVC: *in vitro* culture. Data are Mean ± SEM from six replicates. Values with different superscript letters significantly differed ($P < 0.01$).

3.4.3 TUDCA alters ROS and GSH levels

To explore the antioxidant effects of TUDCA against ER stress-induced impairment of embryonic development, the intracellular ROS and GSH levels in the embryos on Days 2 and 7 of IVC were analyzed. As a result, compared to that the control group, the relative fluorescence intensity of intracellular ROS and GSH levels in embryos on Day 2 were decreased and increased, respectively, in response to TUDCA ($P < 0.01$; Fig. 3.4A and 3.6A). In contrast, the relative fluorescence intensity ROS and GSH levels in embryos at Day 7 did not significantly differ between groups (Fig. 3.4B and 3.6B).

3.4.4 TUDCA suppresses ER stress-related genes expression

To further investigate the mechanism underlying ER stress during early embryogenesis, the expression of ER stress and apoptosis-associated genes in blastocysts at Day 8 were analyzed. The relative expression of ER stress-associated (those encoding *GRP78, IER1*, *CHOP*, *ATF4*, and *ATF6*) genes were reduced by TUDCA treatment compared to that in the control group ($P < 0.05$ and $P < 0.01$; Fig. 3.8A). Moreover, a decrease and an increase in pro-apoptotic *BAX* and anti-apoptotic *BCL2* expression, respectively, were observed in TUDCA-treated embryos compared to that in the control group ($P < 0.01$; Fig. 3.8B). Moreover, the s*XBP1/u*XBP1 ratio was higher in the control group than in the TUDCA group ($P < 0.01$; Fig. 3.9B).

3.4.5 Modulation of ER stress via TUDCA during IVC improves embryo cryo-tolerance

To evaluate whether inhibition of ER stress during culture enhances embryo cryo-tolerance, following culture, blastocysts at Day 8 were subjected to vitrification. As shown in Table 3.3, the embryos treated with TUDCA during IVC had a higher hatching rate compared to

that observed in the control group after 48 h of post-warming (76.9 ± 2.3% vs. 52.5 ± 4.9%; $P < 0.05$). Moreover, despite similar developmental rates, TUDCA treatment showed a lower percentage of apoptotic cells in blastocysts than in the control group (0.44 ± 0.1% vs. 1.42 ± 0.4%; $P < 0.05$). However, no significant difference was found in the total cell number and embryo cleavage rate between groups.

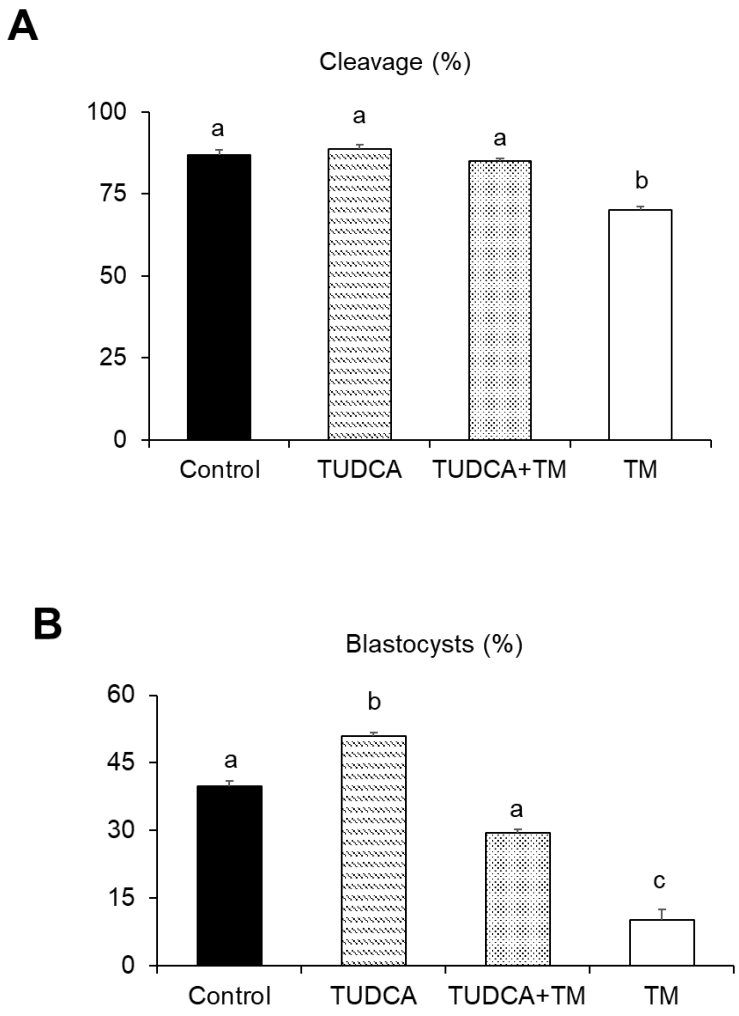

Figure 3.1 Effects of TUDCA and/ or TM during IVC on cleavage (A) and blastocyst (B) developmental rates. Embryos were cultured in IVC medium containing 10 µM TUDCA in the presence or absence of TM (1 µg/mL) until Day 8 of IVC. Values are Mean ± SEM, from seven replicates, and different letters denote significant differences ($P < 0.01$). TUDCA: tauroursodeoxycholic acid; TM: tunicamycin; IVC: *in vitro* culture.

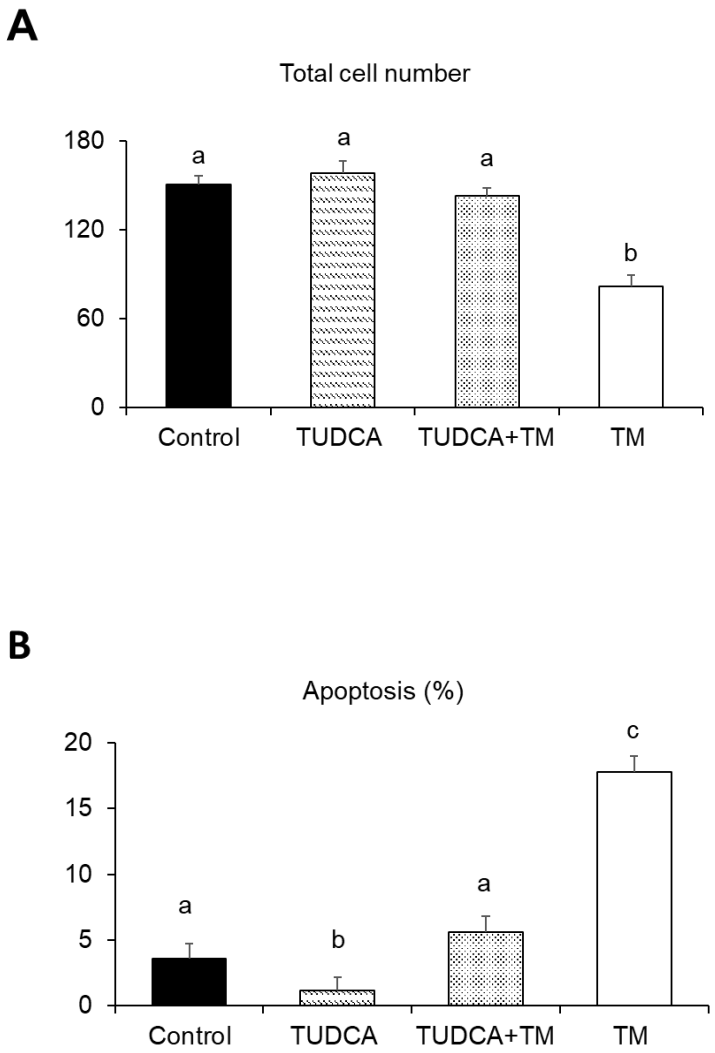

Figure 3.2 Effects of TUDCA and/ or TM during IVC on total cell number (A) and percentage of apoptotic cells (B) in embryos. Embryos were cultured in IVC medium containing 10 μM TUDCA in the presence or absence of TM (1 μg/mL) to Day 8 of IVC. Values are Mean ± SEM, from seven replicates, and different letters denote significant differences ($P < 0.01$). TUDCA: tauroursodeoxycholic acid; TM: tunicamycin; IVC: *in vitro* culture.

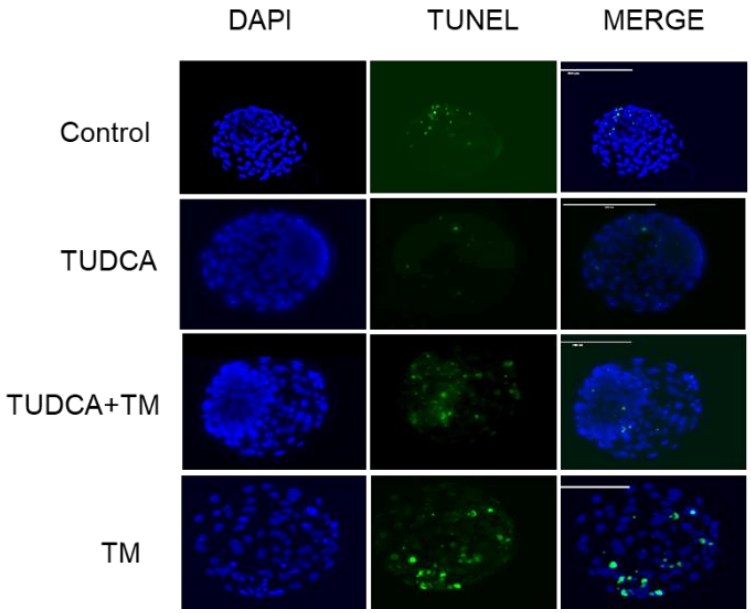

Figure 3.3 Representative images of Day 8 blastocysts applied for TUNEL staining. TUNEL staining was used to detect apoptotic cells, and DAPI staining (blue) was used for nucleus detection. Embryos were cultured in IVC medium containing 10 µM TUDCA in the presence or absence of TM (1 µg/mL) until Day 8 of IVC. TUDCA: tauroursodeoxycholic acid; TM: tunicamycin; DAPI: 4′,6-diamidino-2-phenylindole; IVC: *in vitro* culture; TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

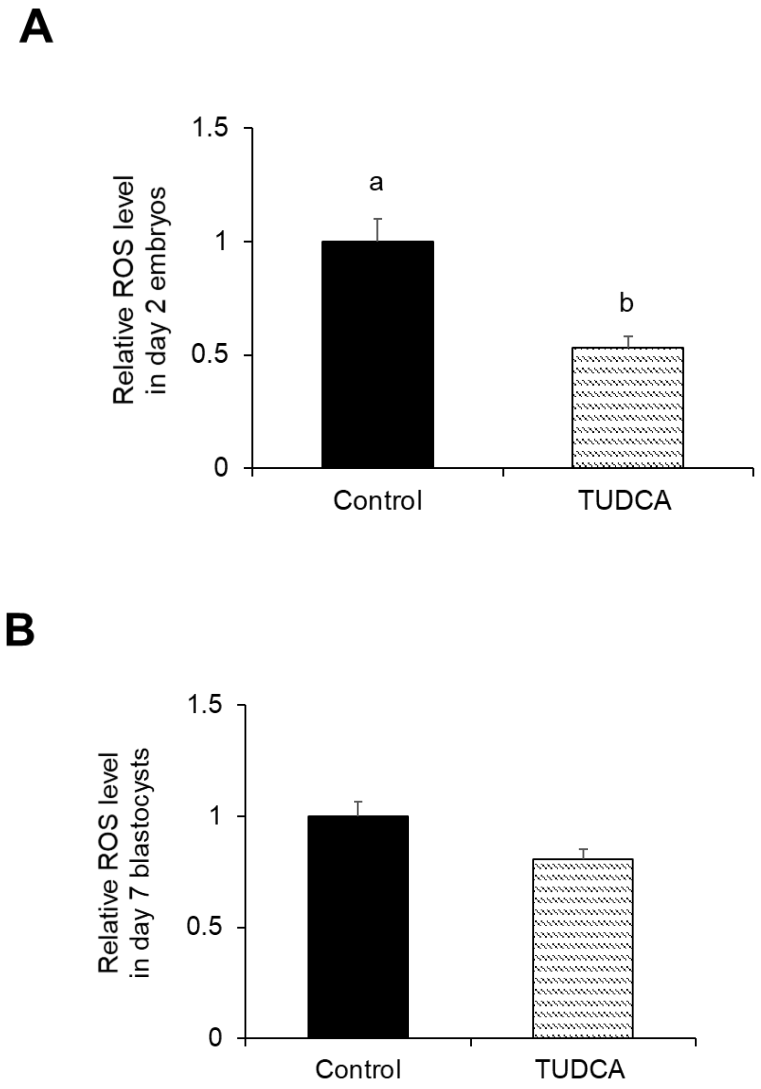

Figure 3.4 Effects of TUDCA on relative fluorescence intensity of intracellular reactive oxygen species (ROS) generation in embryos. Embryos were cultured in IVC medium with or without 10 μM TUDCA. Values are Mean ± SEM from five replicates, and different letters denote significant differences ($P < 0.01$). TUDCA: tauroursodeoxycholic acid; IVC: *in vitro* culture.

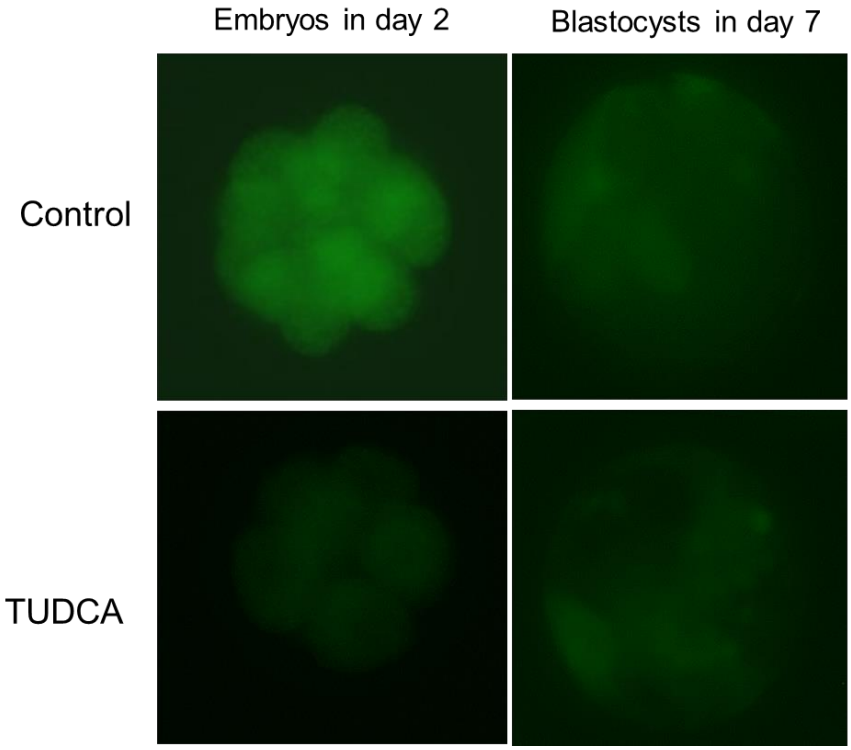

Figure 3.5 Representative images of DCHFDA- labeled embryos obtained from control and TUDCA (10 µM) groups on Days 2 and 7 of IVC. Fluorescence intensity was quantified using Image J software 1.55. TUDCA: tauroursodeoxycholic acid; DCHFDA: 2′,7′-dichlorodihydrofluorescein diacetate; IVC: *in vitro* culture.

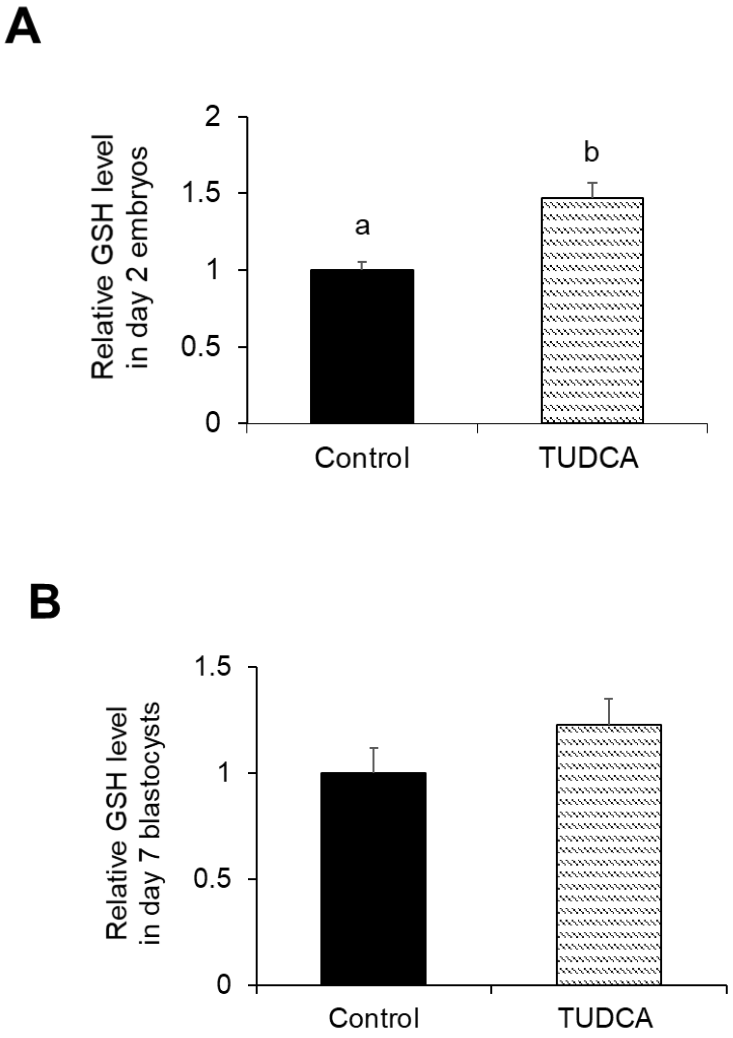

Figure 3.6 Effects of TUDCA on relative fluorescence intensity of reduced glutathione (GSH) level in embryos. Embryos were cultured in IVC medium with or without 10 μM TUDCA. Values are Mean ± SEM from five replicates, and different letters denote significant differences ($P < 0.01$). TUDCA: tauroursodeoxycholic acid, IVC: *in vitro* culture.

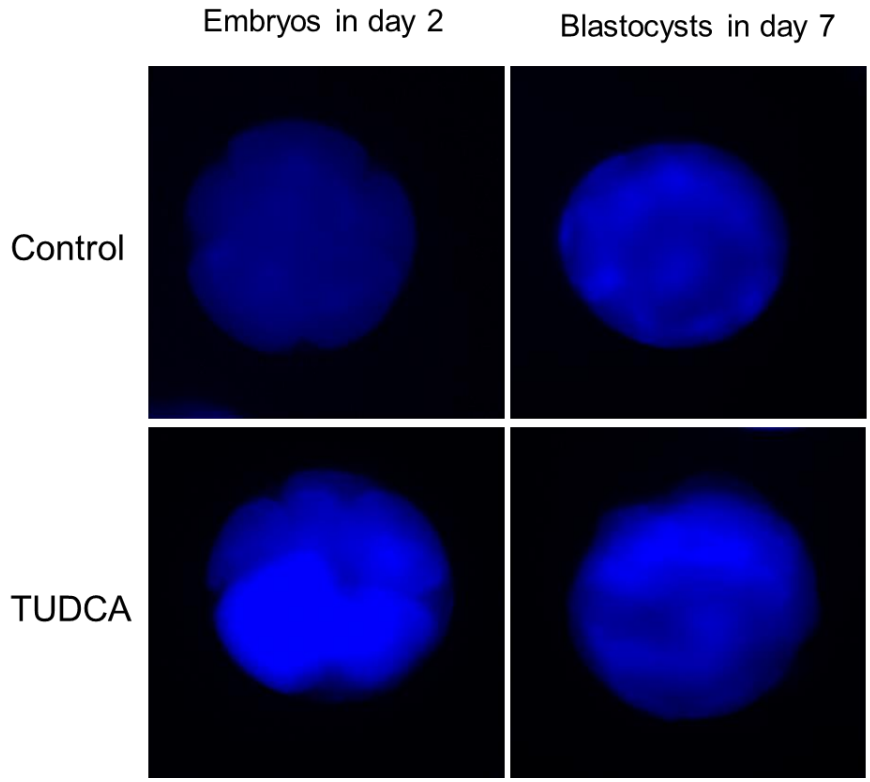

Figure 3.7 Representative images of Cell Tracker Blue (CMF2HC)-labeled embryos obtained from control and TUDCA (10 µM) groups on Days 2 and 7 of IVC. Fluorescence intensity was quantified using Image J software 1.55. TUDCA: tauroursodeoxycholic acid; *CMF2HC*: 4-chloromethyl-6,8-difluoro-7-hydroxycoumarin; IVC: *in vitro* culture.

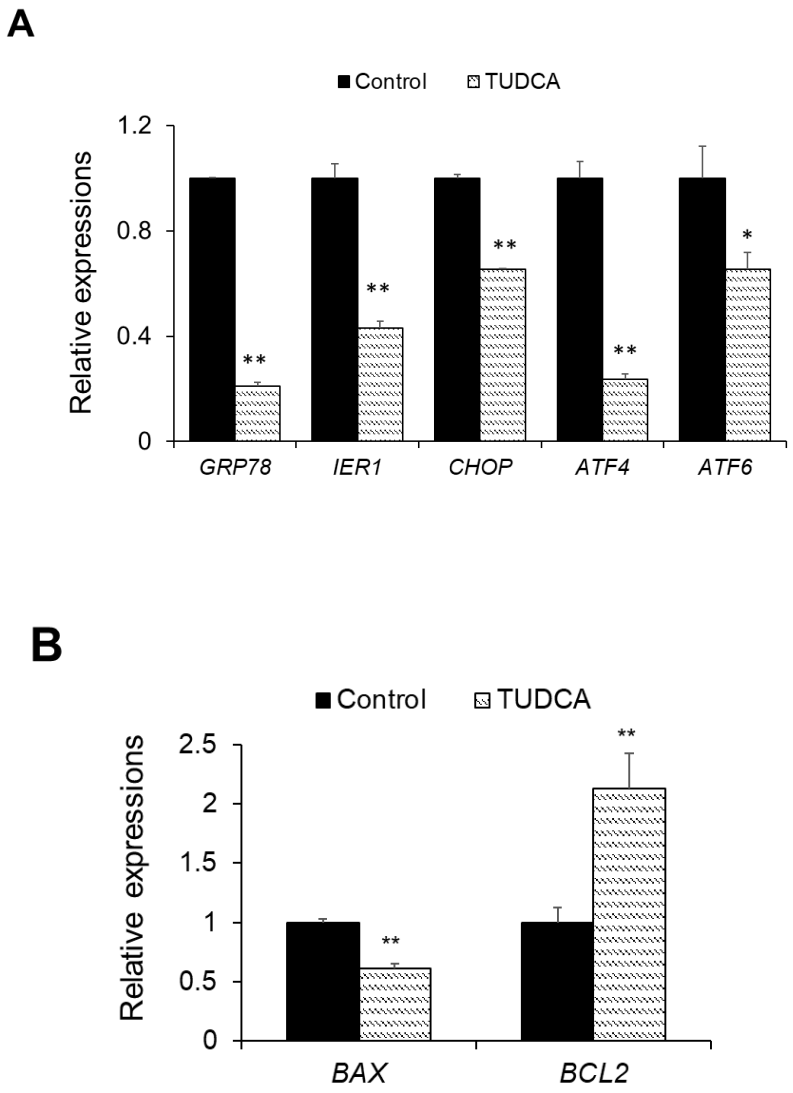

Figure 3.8 Effects of TUDCA on mRNA expression level in embryos. Embryos were cultured in IVC medium with or without 10 μM TUDCA to Day 8 before being analyzed by qPCR. (A, B) The qPCR analysis of the relative abundances of ER-stress- (*GRP78*, *IRE1*, *CHOP*, *ATF4*, and *ATF6*) and apoptosis- (*BAX* and *BCL2*) markers, respectively. Values are Mean ± SEM. **P* < 0.05, ***P* < 0.01. TUDCA: tauroursodeoxycholic acid; IVC: *in vitro* culture; qPCR: quantitative polymerase chain reaction.

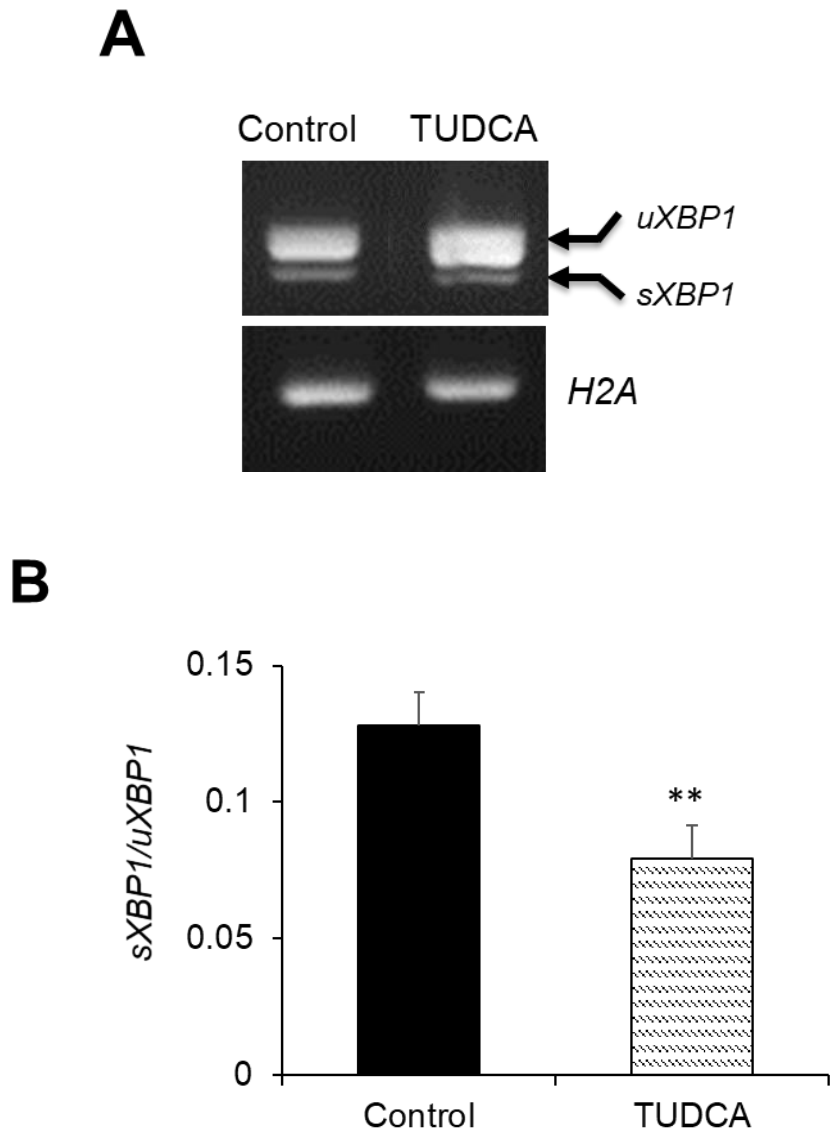

Figure 3.9 Effects of TUDCA on ratio of *sXBP1*/*uXBP1* mRNA expression level, as analyzed by agarose gel electrophoresis. Representative images of expression level of *sXBP1* and *uXBP1* (A) and ratio of *sXBP1* and *uXBP1* (B) mRNA in embryos obtained from control and TUDCA (10 µM) groups on Day 8 of IVC. Values are Mean ± SEM. $^{**}P < 0.01$. Fluorescence intensity was quantified using Image J software 1.55. TUDCA: tauroursodeoxycholic acid; IVC: *in vitro* culture.

Table 3.3 Effects of TUDCA supplementation during IVC on development and quality of vitrified post-warming blastocysts

Treatment	No. of Blastocysts examined	Evaluation on 48 h post-warming			
		Re-expansion (%)	Hatching (%)	Total cell number	Apoptotic cells (%)
0 (Control)	50	87.8 ± 2.8	52.5 ± 8.1[a]	150.1 ± 10.3	1.42 ± 0.4[a]
10 µM TUDCA	50	91.9 ± 3.7	76.9 ± 3.1[b]	159.2 ± 7.5	0.44 ± 0.1[b]

TUDCA: tauroursodeoxycholic acid; IVC: *in vitro* culture. Data are Mean ± SEM from six replicates. Values with different superscript letters significantly differed ($P < 0.01$).

3.5 Discussion

The present study demonstrates that modulating ER stress with TUDCA during IVC influences the developmental chronology and cryo-tolerance of IVP embryos in cattle. The results show that TUDCA at a low dose (10 μM) during IVC increased the developmental competence of bovine embryos, while a high dose (100 μM) had negative effects. A previous study also reported that TUDCA at a high concentration could be toxic to cultured cells (Jia, et al. 2018). It is unclear why high TUDCA concentrations adversely affected cells/embryos. In this study, the decreased cleavage and blastocyst rates recorded in the presence of elevated TUDCA (100 μM) may have been caused by the induction of apoptosis, although this was not assessed. However, these results are in accordance with those obtained in a previous study on cows (Song, et al. 2012).

In this study, the induction of ER stress by TM appears to arrest embryonic development and causes dramatic changes to morphological quality. However, TUDCA effectively reversed these parameters, when TUDCA and TM are administered concurrently. This finding strongly suggests that ER homeostasis is essential during the preimplantation embryonic development. It is well known that TUDCA is an effective ER stress inhibitor and prevents ER-stress-induced cell death (Jia, et al. 2018, Ozcan, et al. 2006, Yun, et al. 2018). The mechanism underlying of this protective property of TUDCA may lie in the presence of a taurine group, which enhances glucose metabolism by stimulating mitochondrial metabolic functions, supports embryonic development, and inhibits apoptosis. TUDCA's anti-apoptotic properties have been reported by many studies. For example, TUDCA prevents BAX translocation and interrupts classical apoptosis pathways by modulating mitochondrial membrane perturbation (Rivard, et al. 2007, Rodrigues, et al.

2000). These findings likely correspond to attenuate ER stress by upregulating *BCL2* and disrupting *BAX* expression according to the results of the present study.

Indeed, ROS production during culture leads to ER stress (Yoon, et al. 2014); and ER stress itself can produce ROS (Landau, et al. 2013). This mechanistic link between ROS and ER stress is associated with mitochondrial dysfunction. In the present study, TUDCA let up ER stress by decreasing ROS production. TUDCA has strong antioxidant properties that can mitigate ROS generation and directly suppress oxidative stress and subsequent ER stress (Yoon, et al. 2014, Zhang and Wang 2018). TUDCA affects Akt-mediated MnSOD activation, which protects cells against ROS (Yun, et al. 2018). In the present study, TUDCA treatment significantly increased the level of GSH, which is a substrate of glutathione peroxidase and is the primary antioxidant enzyme (Luberda 2005) in embryos. In addition, ROS and GSH levels in embryos at Day 7 did not significantly differ between the groups, suggesting that ER stress is mainly induced at an early stage of development, and that TUDCA could mitigate ROS production by increasing GSH production.

TUDCA is frequently associated with the modulation of the ER stress-induced UPR. Molecules involved in the UPR signaling pathways, such as *GRP78*, *PERK*, *IRE1*, *ATF4*, *ATF6*, *XBP1*, and *CHOP*, are mainly induced by ER stress and are considered biomarkers of ER stress (Kadowaki and Nishitoh 2013, Park, et al. 2013, Zhang and Wang 2018). In this study, higher mRNA expression level of *GRP78*, *IER1*, *CHOP*, *ATF4*, and *ATF6* in the control embryos resulted in the existence of ER stress during IVC and cellular damage induced by ER stress. The presence of this enhancement in ER stress is further supported by the observation of elevated level of *sXBP1* in control embryos. Because, *sXBP1* is induced by IER1 endonuclease activity under ER stress (Lee, et al. 2003). *GRP78* is an

intended regulator of embryonic development and a marker of ER stress (Luo, et al. 2006, Zheng, et al. 2014). In this study, the expression of these genes was decreased in embryos treated with TUDCA, indicating that ER stress-induced UPR signal is inhibited by TUDCA during culture. These findings also supported by the downregulation of *BAX* and *CHOP* expression in embryos treated with TUDCA in the present study. Because these apoptosis-related molecules directly interact with the cytosolic domain of IRE1 under excessive ER stress (Hetz, et al. 2006). Nevertheless, these results suggest that ER stress-mediated UPR activation may play a key role in poor developmental competence of embryos.

However, it is unknown whether TUDCA provides the cryo-tolerance capability to the embryo during culture or not. Growing evidence suggests that improving post-fertilization culture conditions increases embryo quality (Lonergan, et al. 2003), and embryos with rapid developmental kinetics during culture survive cryo-preservation better than slower ones (Nedambale, et al. 2004). We observed that TUDCA during IVC improved embryo cryo-tolerance following vitrification, and significantly increased the hatching rate and reduced the number of apoptotic cells at 48-h post-warming culture compared to the control group. This finding is essential because cryo-preservation hardens the zona pellucida, thereby preventing the blastocyst hatching process after embryo transfer (Carroll, et al. 1990, Matson, et al. 1997). A high hatching rate after cryo-preservation is a reliable indicator of embryo quality. Hence, it is probable that the beneficial effects that TUDCA has on blastocyst cryo-tolerance relies upon embryo quality obtained during IVC. One of the notable effects of TUDCA is to maintain the embryonic redox state through its strong antioxidant activity and mitochondrial functions, as shown by the decreased ROS and apoptosis levels. It is reasonable to predict that TUDCA improves embryonic cryo-tolerance by scavenging ROS, which increases embryonic antioxidant capability during culture.

Therefore, the novelty of this study is that controlling ER stress via TUDCA during IVC increases embryonic developmental chronology and cryo-tolerance in cattle. The findings of this study improve our understanding of the early developmental process of embryos and may lead to transfer high-quality embryos produced *in vitro* under commercial settings in mammals. Thus, data have important implications in the field of assisted reproduction and on future studies that investigate the mechanisms underlying the beneficial effects of TUDCA on development to term after the transfer of vitrified embryos.

Chapter 4

Inhibition of endoplasmic reticulum stress during *in vitro* maturation enhances the developmental potential of bovine oocytes

4.1 Abstract

In chapter 3, the results show that regulation of ER stress during IVC acutely increases bovine embryo developmental rate and cryotolerance; these data indicate that ER stress is a critical factor reducing the quality of IVP embryos. This follow-up study examined whether ER stress attenuation during IVM influences meiotic maturation, oocyte quality, and subsequent embryonic development. Bovine COCs obtained from slaughterhouse ovaries were matured with or without TUDCA (0, 50, 100, and 200 μM), a selective inhibitor of ER stress for 22 h followed by IVF, and embryos were cultured for 8 days. The dose-dependent experiment showed that 100 μM TUDCA significantly increased the maturation rate, and decreased ROS in denuded oocytes, and appeared lower number of apoptotic cells in matured COCs. Subsequently, treatment of TUDCA (100 μM) decreased the localization and amount of GRP78/BIP protein level as well as ER stress- (*GRP78/BIP, PERK, IER1, ATF4,* and *XBP1)* and apoptosis (*CHOP* and *BAX*)- related gene expression; while it increased the anti-apoptotic gene *BCL2* level in matured COCs. Moreover, addition of TUDCA (100 μM) during IVM significantly improved the blastocyst formation rate (43.6 ± 1.8% vs. 49.7 ± 1.3%) and decreased the number of apoptotic cells (7.7 ± 1.1% vs. 5.03 ± 0.6%) in blastocysts. These findings suggest that the presence of ER stress during maturation impairs the developmental competence of bovine COCs, and that this process can be reversed by TUDCA.

4.2 Introduction

Over the world, one of the greatest challenges for the researcher is to mimic *in vivo* conditions for *in vitro* assisted reproductive technology. A low-efficiency IVM of oocytes has emerged as a crucial issue for poor developmental competence of embryos produced *in vitro* (Lonergan, et al. 2006). Only 30-40% oocytes reach into blastocysts stage following IVM, whereas 70% of *in vivo* matured oocytes develop into blastocysts under *in vitro* conditions (Rizos, et al. 2002). The lack of development of these oocytes is probably due to an impaired cytoplasmic maturation, which is essential for ultrastructural and functional modifications of oocytes that allow them to be fertilized and subsequent embryonic development (Reader, et al. 2017, Rizos, et al. 2002). A recent study disclosed that use of *in vivo*-matured oocytes collected from superstimulated Japanese Black Cows enhance the quality of blastocysts produced *in vitro* compared with the *in vitro* matured oocytes (Egashira, et al. 2019). Therefore, it is necessary to understand the processes involved in maturation of the COCs *in vitro.*

Recent evidences suggest that stress to ER is a major fact for poor developmental competence of oocyte maturation and embryo development *in vitro* (Acton, et al. 2004, Lin, et al. 2019a, Wakefield, et al. 2008, Wu, et al. 2010). Although, the exact molecular mechanisms of ER stress remain poorly described in mammalian embryo development, it is generally accepted that occurrence of ER stress can be detected by the expression of the UPR pathway mediators: PERK, ATF6, and IRE1 (Arensdorf, et al. 2013, Tang and Yang 2015). A direct evidence is the observation in chapter 3 in this study, where the induction of ER stress during IVC apparent increase of ER stress-induced UPR signaling molecules and interferes the developmental processes of bovine embryos that can be reversed by TUDCA, an ER stress inhibitor. Many studies either *in vitro* or *in vivo* demonstrated that

TUDCA functions as a chemical chaperone; attenuates ER stress by preventing UPR dysfunction, and stabilizing mitochondria (Chen, et al. 2008, de Almeida, et al. 2007, Vang, et al. 2014). However, ER stress-induced dysfunction has been well observed in mice and pigs, but little is known about its effects on bovine *in vitro.*

Therefore, the objective of the present study was to determine whether the attenuation of ER stress during IVM improves the developmental competence of bovine oocytes.

4.3 Materials and methods

4.3.1 Oocyte collection and IVM

Good quality COCs were aspirated from bovine ovaries obtained from a local abattoir and IVM was conducted based on the methods described in chapter 2 (see section 2.3.2).

4.3.2 Assessment of meiotic maturation

After 22 h of IVM, meiotic maturation was assessed by nuclear stages. Oocytes were denuded by pipetting in TCM-199 medium containing 0.1% hyaluronidase, washed with PBS containing 0.5 mg/mL PVP and mounted on microscope slides. The samples were fixed for 2–3 days with 25% (v/v) acetic acid in ethanol and stained with 1% acetic orcein (w/v) in 45% (v/v) acetic acid for 60 min. Meiotic stages of the oocytes were evaluated under a microscope (Eclipse Ti, Nikon, Tokyo, Japan).

4.3.3 Culture treatment

To modulate ER stress, COCs were matured in IVM medium with the ER stress inhibitor TUDCA (sodium salt). TUDCA was provided as a lyophilized powder, dissolved in distilled water to create 50 mM stock solutions, and stored at 4 °C until further use for up to 2 wk. Next, the stock solution was diluted in IVM medium to obtain 50-, 100-, and 200-μM TUDCA solutions.

4.3.4 Assessment of oxidative stress

Oxidative stress as assessed by intracellular ROS in matured denuded oocytes based on the procedures described in chapter 3 (see section 3.3.4).

4.3.5 Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining

A TUNEL assay kit (in situ cell death detection kit; Roche, Basel, Switzerland) was used to evaluate the presence of apoptotic cells in matured COCs based on the methods described in chapter 2 (see section 2.3.5).

4.3.6 qPCR analysis

The RNA extraction and qPCR were carried out according to procedures described in chapter 2. Briefly, total RNA (40 COCs per sample) was isolated using a RNeasy Micro Kit according to the manufacturer's instructions. Extracted RNA was either used immediately for RT or stored at −80 °C. The RT was performed using the SuperScript™ IV VILO™ Master Mix cDNA Synthesis Kit according to the manufacturer's instructions. The RT mix (20 µL) consisted of 12 µL RNA, 4 µL SuperScript™ IV VILO™ Master Mix, and 4 µL nuclease-free water. The RT reaction was performed with annealing of random primers (10 min at 25 °C), cDNA synthesis (10 min at 50 °C), and termination of the reaction (5 min at 85 °C). Each sample was analyzed in duplicate using the SsoAdvanced™ universal SYBER® green supermix with CFX Connect. The real-time PCR mix (20 µL) consisted of 2 µL cDNA, 10 µL supermix, 6 µL nuclease-free water, and 1 µL each of forward and reverse primers per gene. The program used for the amplification included a denaturing cycle (30 s at 95 °C), 40 cycles of PCR (95 °C for 5 s and 58 °C for 5 s), and a melting curve program (60–95 °C with a heating rate of 0.5 °C/s, continuous fluorescence acquisition, and cooling to 30 °C). The fold changes of target genes were evaluated by the ΔΔCT method using endogenous reference gene (*RN18S1*) expression. The PCR primers utilized to amplify each gene are listed in Table 4.1.

Table 4.1 List of primers used for PCR analysis

Gene Name	Primer Sequence (5′–3′)	Product Size (bp)	Accession number
BAX	F: ctactttgccagcaaactgg R: tcccaaagtaggagagga	158	NM_173894.1
CASPASES 3	F: cccaagtgtgaccactgaac R: ccattaggccacactcactg	169	NM_001077840
PERK	F: ggctgaaagatgacagcaca R: agaactggctctcggatgaa	195	NM_001098086.1
GRP78/BIP	F: tgcagcaggacatcaagttc R: tttgtttgcccacctccaac	91	NM_001075148.1
XBP1	F: tagcagctcagactgccaga R: attccccttggtctctgctt	157	NM_001271737.1
BCL2	F: tggatgaccgagtacctgaa R: gagacagccaggagaaatcaaa	123	NM_001166486.1
ATF6	F: tgaacttcgaggatgggttc R: gaatttgagccctgttccag	117	NM_001075935
ATF4	F: ggccaagcacttcaaacatc R: aagcatcctccttgctgttg	110	NM_001034342.2
CHOP	F: gcaccaagcatgaacagttg R: atcgatggtggttgggtatg	116	NM_001078163.1
IRE1	F: ccgaagttcagatggcattc R: tctgcaaaggctgatgacag	108	XM_001789477.1
RN18S1	F: aaacggctaccacatccaagg R: gcggaaggatttaaagtggactc	138	DQ066896

4.3.7 Immunohistochemical detection of GRP78/BIP protein

After maturation, COCs were washed three times in 0.1% (w/v) PVP-PBS and fixed with 4% paraformaldehyde in PBS for 40 min at room temperature (RT), followed by three washes with 0.1% (w/v) PVP-PBS. Then COCs were permeabilized with 0.1% (w/v) PVP-PBS containing 0.5% TritonX-100 for 30 min at RT and blocked in blocking solution (2 % (w/v) BSA in 0.1% (w/v) PBS-PVA) for 1 h. The samples were incubated with the primary anti-GRP78/BIP (1:200; Cat. No. MA5-27686, Invitrogen, Canada) antibody at 4°C overnight, washed three times with 0.1% (w/v) PVP-PBS containing 0.1% (v/v) Tween-20 (Cat. No. 166-2115, FUJIFILM Wako Pure Chemical Corporation) for 10 min and then reacted with the anti-mouse fluorescein isothiocyanate (FITC)-conjugated IgG (1:150; Lot No. 064M4788V, Sigma) secondary antibody at RT under dark condition. Primary and secondary antibodies were diluted with Can Get Signal® Immunostain Immunoreaction Enhancer Solution A (TOYOBO, NKB-501, Osaka, Japan). COCs were mounted on slides using mounting medium with DAPI and examined under a confocal laser microscopy (C1, Nikon, Tokyo, Japan, Excitation: 488 nm, Emission 515 nm).

4.3.8 Western blot analysis

Maturated COCs (40 per sample) were washed three times with PBS and lysed in 40 µL lysis buffer (Bolt LDS Sample Buffer, Thermo Fisher Scientific) and subsequently denatured at 70 °C for 10 min. The protein samples were separated by electrophoresis in Bolt^{TM} 4–12% Bis-Tris Plus SDS polyacrylamide precast gels (Invitrogen, Carlsbad, CA, USA) and transferred to PVDF membranes using an iBlot®2 Dry Blotting System (Thermo Fisher Scientific). The membranes were blocked by incubation in PVDF blocking reagent (Cat. No. B1140201, Toyobo) for 1 h and then washed three times with EzTBS (Cat. No. WSE-7230, ATTO, Tokyo, Japan) containing 0.5% (v/v) Tween-20 (TBS-T) at room

temperature. Membranes were incubated with primary anti-GRP78/BIP (1:2000; Cat. No. MA5-27686, Invitrogen, Canada) and anti-β-actin (1:5000; Cat. No. 010-27841, FUJIFILM Wako Pure Chemical Corporation) antibodies at 4 °C overnight. On the next day, after three washes with TBS-T, membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (1:30000 dilution; Cat. No. NA931, GE Healthcare) at 37 °C for 1 h. Primary and secondary antibodies were diluted with immunoreaction enhancer (Can Get Signal; Toyobo). Membranes were washed extensively with TBS-T before bound antibody detection using the ECL-Prime Western Blotting Detection system (GE Healthcare) as per the manufacturer's instructions. Immunoreactive signals were captured and analyzed utilizing NIH image software 1.55 (National Institutes of Health, Bethesda, MD, USA).

4.3.9 *In vitro* embryo production

In vitro embryo production was carried out according to the procedure described in chapter 2 (see section 2.3.3).

4.3.10 Experimental design

Experiment 1: Effects of different concentrations of TUDCA on meiotic maturation and ROS content of oocytes

To assess the dose-dependent effects of TUDCA during IVM, bovine COCs were cultured in maturation medium supplemented with different concentrations (0, 50, 100, and 200 µM) of TUDCA at 38.5 °C for 22 h in a humidified atmosphere of 5% CO_2 in air and investigated the maturation rate of oocytes by acetic-orcein staining, and the intracellular ROS content in denuded oocytes by DCHFDA fluorescence. Moreover, denuded oocytes cultured either

with or without TUDCA (100 µM) during IVM and after maturation ROS level was detected to know the effect of ER stress in cumulus cells or oocytes.

Experiment 2: Effects of TUDCA on the apoptosis status of COCs

To investigate the effects of TUDCA in reducing ER stress and oocyte quality, COCs were matured with different doses of TUDCA (0, 50, 100, and 200 µM) and apoptotic cells were detected by using TUNEL assay. Additionally, qPCR analysis was performed to examine apoptosis-associated gene expression in COCs matured with or without TUDCA (100 µM).

Experiment 3: Immunohistochemical and western blot analysis of ER stress-induced GRP78/BIP protein in matured COCs

To confirm the presence of ER stress and efficacy of TUDCA against ER stress, the localization and amount of ER chaperone GRP78/BIP protein was detected by both immunohistochemical and western blot analysis in matured COCs obtained from control and TUDCA groups.

Experiment 4: Effects of TUDCA on ER stress-induced genes expression in COCs

To examine the inhibitory effects of TUDCA on ER stress and its relation to maturation, the expression of ER stress-associated genes (those encoding *GRP78/BIP, PERK, IER1, ATF4, ATF6, CHOP*, and *XBP1)* in COCs matured with or without TUDCA (100 µM) were analyzed by using qPCR.

Experiment 5: The effects of TUDCA during IVM on subsequent embryonic development *in vitro*

To assess the effects of ER stress during IVM on subsequent embryonic development, COCs were matured either with or without TUDCA (100 μM) and subjected to fertilization followed by embryo culture for 8 days. Each trial of experiment run was accompanied by parallel control with the same sample and cultural conditions. The developmental competence of embryos was examined by cleavage and blastocyst rates on Days 2 and 8, respectively. The total cell number and apoptotic status of the blastocysts on Day 8 were also determined by using TUNEL assay to investigate embryo quality.

4.3.11 Statistical analysis

For each experiment, at least three independent replicates were performed unless specified otherwise. Data are presented as mean ± SEM. All statistical analyses were carried out using BellCurve for Excel version 3.20 (Social Survey Research Information, Tokyo, Japan). Differences between two groups were assessed by Student's t-test. Differences among three or more groups were examined using ANOVA followed by the Tukey-Kramer multiple comparison test. All percentage data were arcsine transformed prior to statistical analysis. Differences were considered significant at $P < 0.05$ and $P < 0.01$.

4.4 Results

4.4.1 Effects of TUDCA on the maturation rate and ROS level of oocytes

The dose-dependent treatment of IVM culture with TUDCA indicated that the 100 μM dose added to the maturation medium is effective for ER stress control, since a significantly higher percentage of oocytes progressed to metaphase II (MII) stage in the 100 μM TUDCA than in the control group (89.4 ± 1.4% vs. 79.5 ± 2.6%; $P < 0.01$; Table 4.2). Consequently, 100 μM TUDCA treatment significantly decreased the relative fluorescence intensity of ROS content in comparison with the control group ($P < 0.01$; Fig. 4.1). However, the percentage of MII-stage oocytes (Table 4.2) and the relative fluorescence intensity of the ROS content (Fig. 4.1) were similar among the control, 50, and 200 μM TUDCA groups. No significant differences were observed in the percentage of GV, GVBD, and metaphase I (MI)-stage oocytes among the groups (Table 4.2).

4.4.2 Effects of TUDCA on the incidence of apoptosis in COCs

Based on the TUNEL assay results, the presence of TUNEL-positive cells in the 100 μM TUDCA group apparently decreased compared with that in other groups (Fig. 4.2A). Furthermore, qPCR analysis revealed that treatment of COCs with 100 μM TUDCA significantly decreased the pro-apoptotic gene *BAX* and increased the anti-apoptotic gene *BCL2* expression compared with the control group ($P < 0.05$; Fig. 4.2B). However, no significant change was observed for *CASPASE 3* expression among the groups (Fig. 4.2B).

4.4.3 Effects of TUDCA on GRP78/BIP protein expression in COCs

As shown in Figure 4.3A, both the number of GRP78/BIP protein aggregates and the fluorescence intensity in the untreated COCs were appeared higher than in the COCs treated with TUDCA (100 μM). In addition, the results of western blot analysis clearly revealed a

significant decrease (P <0.01) of GRP78/BIP protein level in COCs treated with TUDCA (100 μM) in comparison with the control group (Fig. 4.3B and C).

4.4.4 Effects of TUDCA on ER stress-associated gene expression in COCs

The qPCR analysis clearly revealed that addition of 100 μM TUDCA to the maturation medium significantly decreased the expression of ER stress-associated genes (*GRP78/BIP*, *PERK*, *ATF4*, *IER1*, *XBP1*, and *CHOP)* in COCs in comparison with the control group (P < 0.01; P < 0.05; Fig. 4.4). However, TUDCA treatment had no significant effect on *ATF6* gene expression (Fig. 4.4).

4.4.5 Effects of TUDCA treatment during IVM on the subsequent embryo development *in vitro*

To determine the effects of TUDCA on inhibition of ER stress during IVM, matured COCs were subjected to fertilization followed by embryo culture. ER stress during IVM significantly decreased the quality and blastocyst developmental rate, whereas the addition of 100 μM TUDCA to the maturation medium significantly improved the blastocyst formation rate (43.6 ± 1.8% vs. 49.7 ± 1.3%; P < 0.05; Fig. 4.5A) and decreased the percentage of TUNEL-positive cells in blastocysts (7.7 ± 1.1% vs. 5.03 ± 0.6%; P < 0.05; Fig. 4.6B). However, no significant differences were found in the cleavage rate and total cell number in embryos between the groups (Fig. 4.5A and 4.6A).

Table 4.2 Effects of TUDCA supplementation during *in vitro* maturation of bovine oocytes.

TUDCA (μM)	No. of oocytes examined	Meiosis Development (%)			
		GV	GVBD	MI	MII
0 (Control)	232	1.9 ± 0.6	4.3 ± 1.5	14.3 ± 2.1	79.5 ± 2.6[a]
50	232	1.2 ± 0.6	4.9 ± 1.1	14.7 ± 1.9	81.1 ± 0.9[a]
100	239	1.0 ± 0.5	1.4 ± 0.7	8.1 ± 1.1	89.4 ± 1.4[b]
200	236	1.8 ± 0.7	7.1 ± 2.2	14.2 ± 2.3	76.9 ± 3.4[a]

Bovine oocytes were matured with different concentrations of TUDCA for 22 h, and the meiotic maturation rate was examined. TUDCA: tauroursodeoxycholic acid; GV: germinal vesicle; GVBD: germinal vesicle breakdown; MI: metaphase I; MII: metaphase II. Data are mean ± SEM. from seven replicates. [a, b]Values with different superscripts within the same column are significantly different ($P < 0.01$).

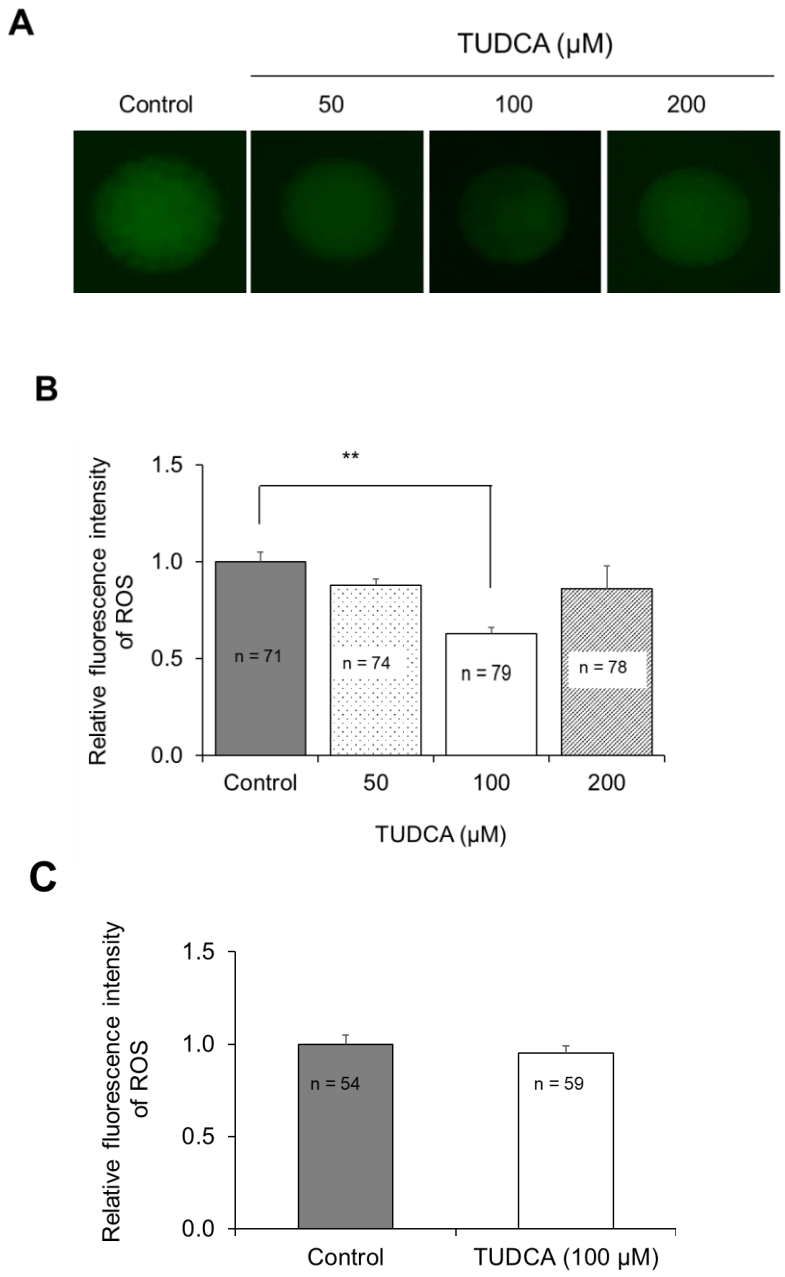

Figure 4.1 Effects of TUDCA on intracellular ROS generation in oocytes during maturation. (A) Representative images of ROS fluorescence (green) and (B and C) quantification of the relative ROS levels in denuded oocytes, respectively. During IVM, COC matured with TUDCA (Fig. B) and denuded oocyte matured with TUDCA (Fig. C). Data are means ± SEM from three replicates. Values in bars represent the number of blastocysts examined in each group. ** denotes significant differences ($P < 0.01$). TUDCA: tauroursodeoxycholic acid; ROS: reactive oxygen species.

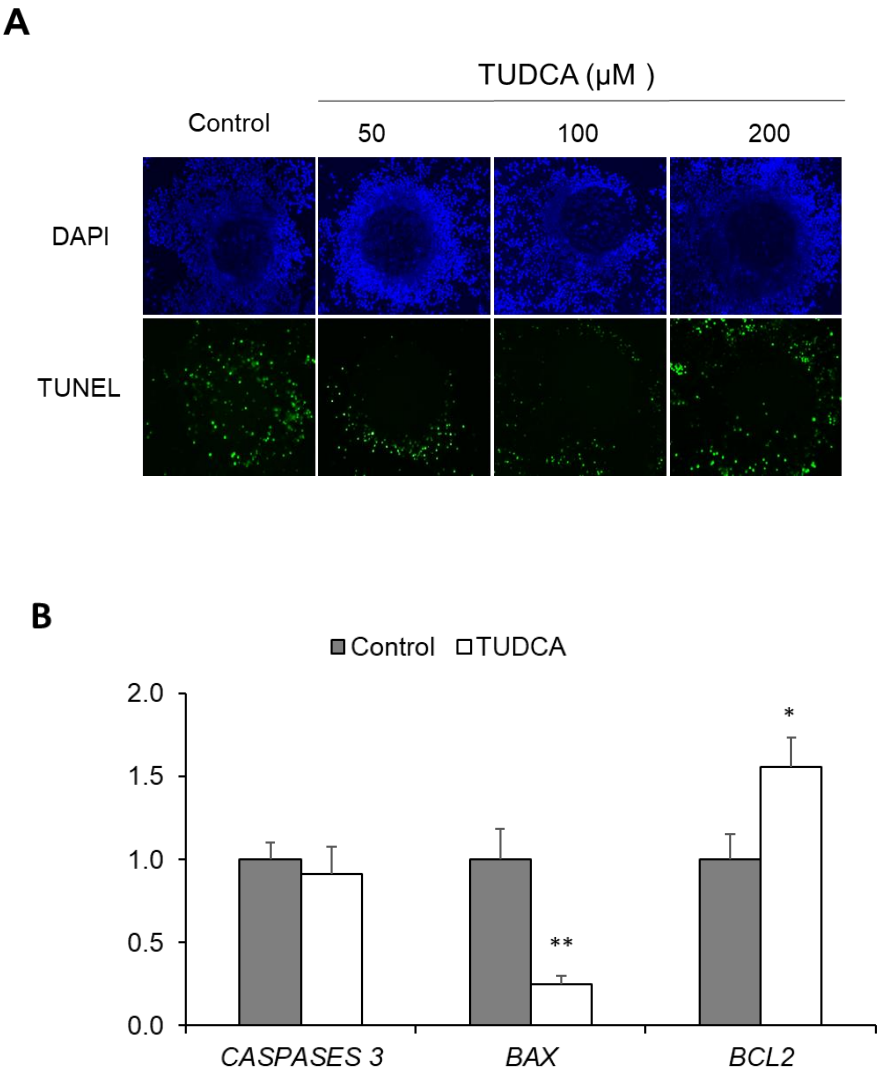

Figure 4.2 Effects of TUDCA on apoptosis of matured COCs. (A) Representative images of COCs applied for TUNEL staining from four different groups. TUNEL (green) and DAPI (blue) staining were used for apoptotic cells and DNA detection, respectively. (B) The relative expression of *CASPASES 3*, *BAX* and *BCL2* in COCs obtained from control and 100 μM TUDCA group by quantitative real-time PCR. Values are means ± SEM from four independent experiments. *: $P < 0.05$; **: $P < 0.01$. TUDCA: tauroursodeoxycholic acid; COCs: cumulus oocyte complexes; PCR: polymerase chain reaction; DAPI: 4′,6-diamidino-2-phenylindole; TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

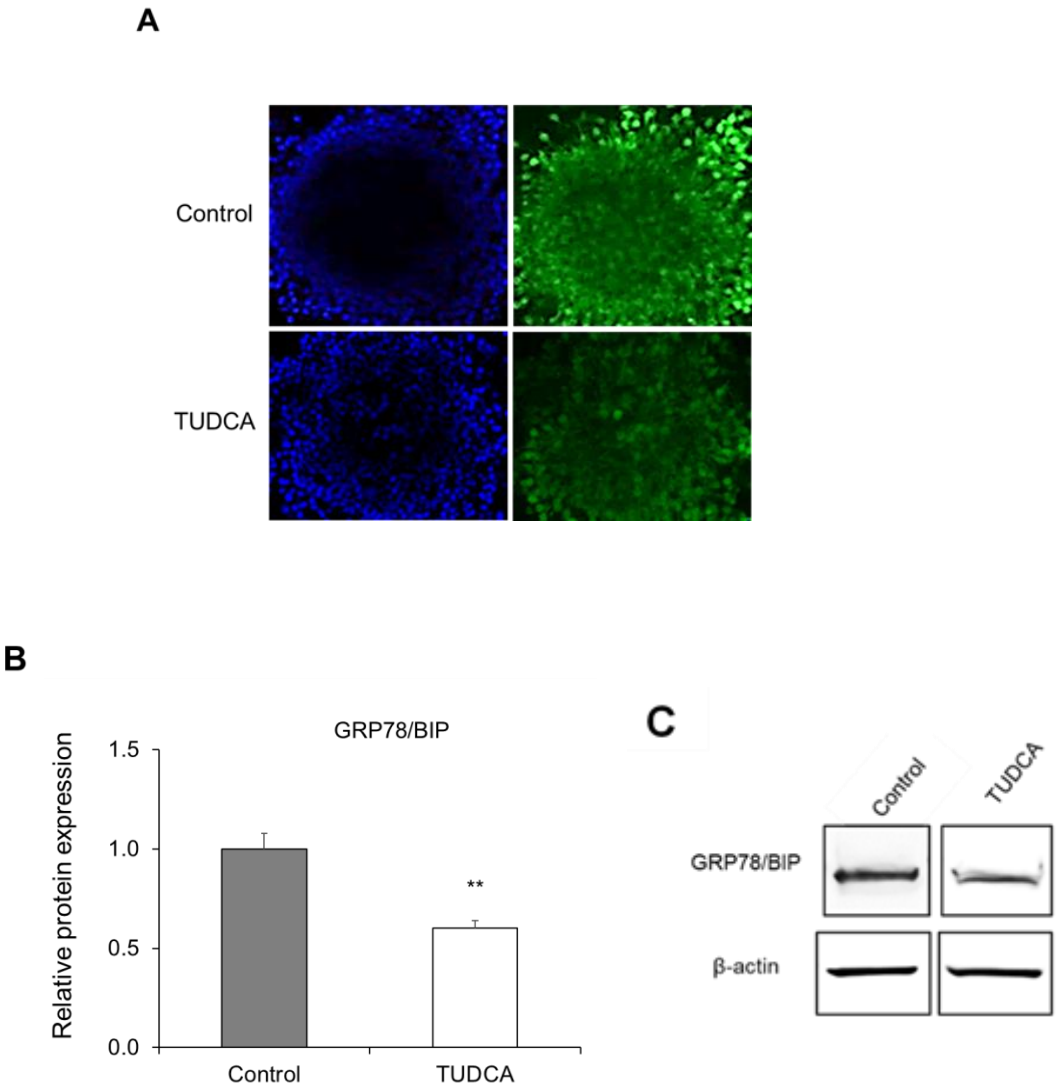

Figure 4.3 Effects of TUDCA on the localization and amount of GRP78/BIP protein level in COCs. (A) Immunohistochemical detection of GRP78/BIP protein in matured COCs. (B and C) GRP78/BIP protein and relative expression levels in COCs determined by western blot analysis (five replicates). Values are means ± SEM and marked with asterisks groups differ significantly (**P < 0.01). COCs: cumulus cell complexes; TUDCA: tauroursodeoxycholic acid.

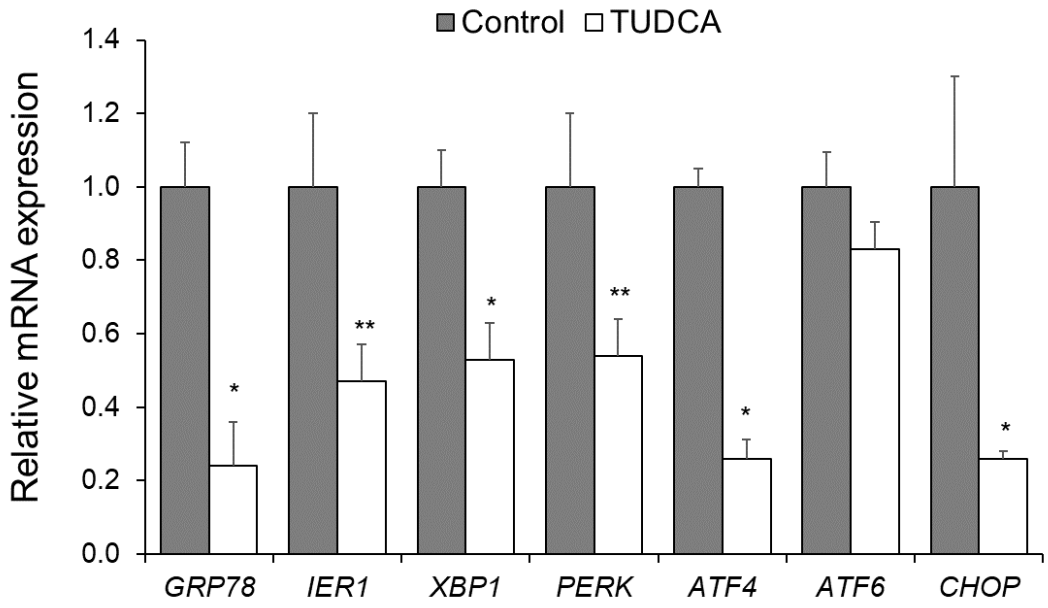

Figure 4.4 Effects of TUDCA on the relative expression of endoplasmic reticulum stress-associated genes (*GRP78, PERK, ATF4, ATF6, IRE1, XBP1,* and *CHOP*) in COCs measured by qPCR (three replicates) after treated either with or without TUDCA (100 μM) during maturation. Values are means ± SEM and marked with asterisks groups differ significantly (*P < 0.05; **P < 0.01). COCs: cumulus cell complexes; TUDCA: tauroursodeoxycholic acid; qPCR: quantitative polymerase chain reaction.

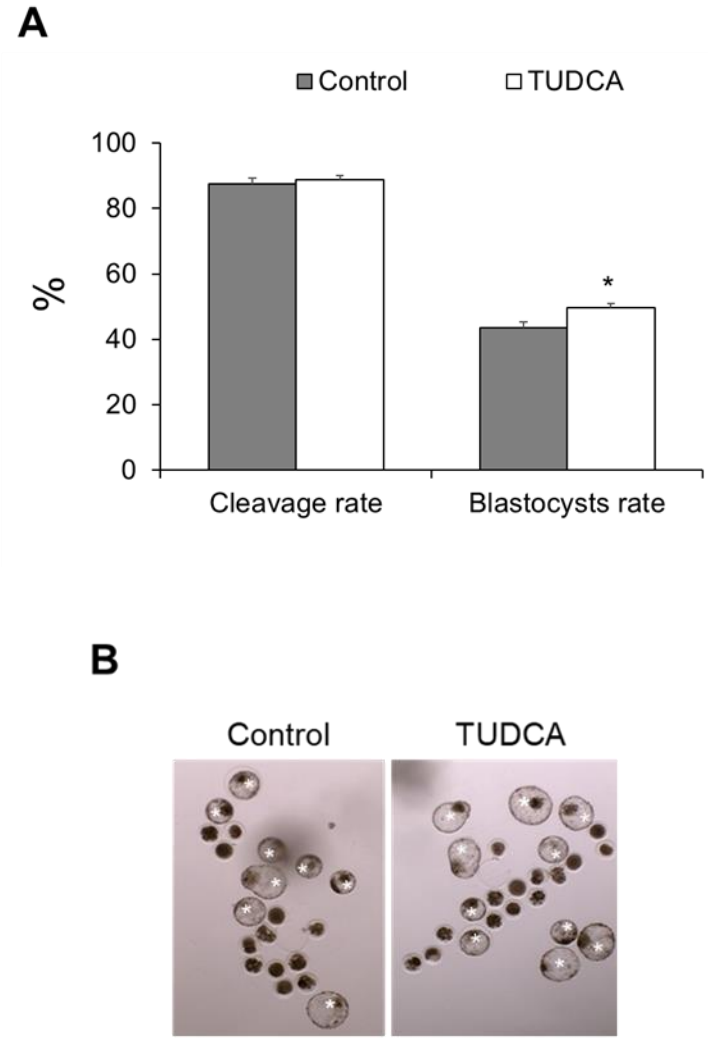

Figure 4.5 Effects of TUDCA during IVM on the developmental competence of oocytes. Bovine COCs were cultured in the presence or absence of TUDCA (100 μM) during IVM followed by *in vitro* fertilization and embryo culture for 8 days. (A) The cleavage and blastocyst developmental rates were examined on Days 2 and 8, respectively. (B) Representative images of embryos at the blastocyst stage marked by a white star (*). The values are means ± SEM from five independent experiments. *: $P < 0.05$. TUDCA: tauroursodeoxycholic acid; IVM: *in vitro* maturation.

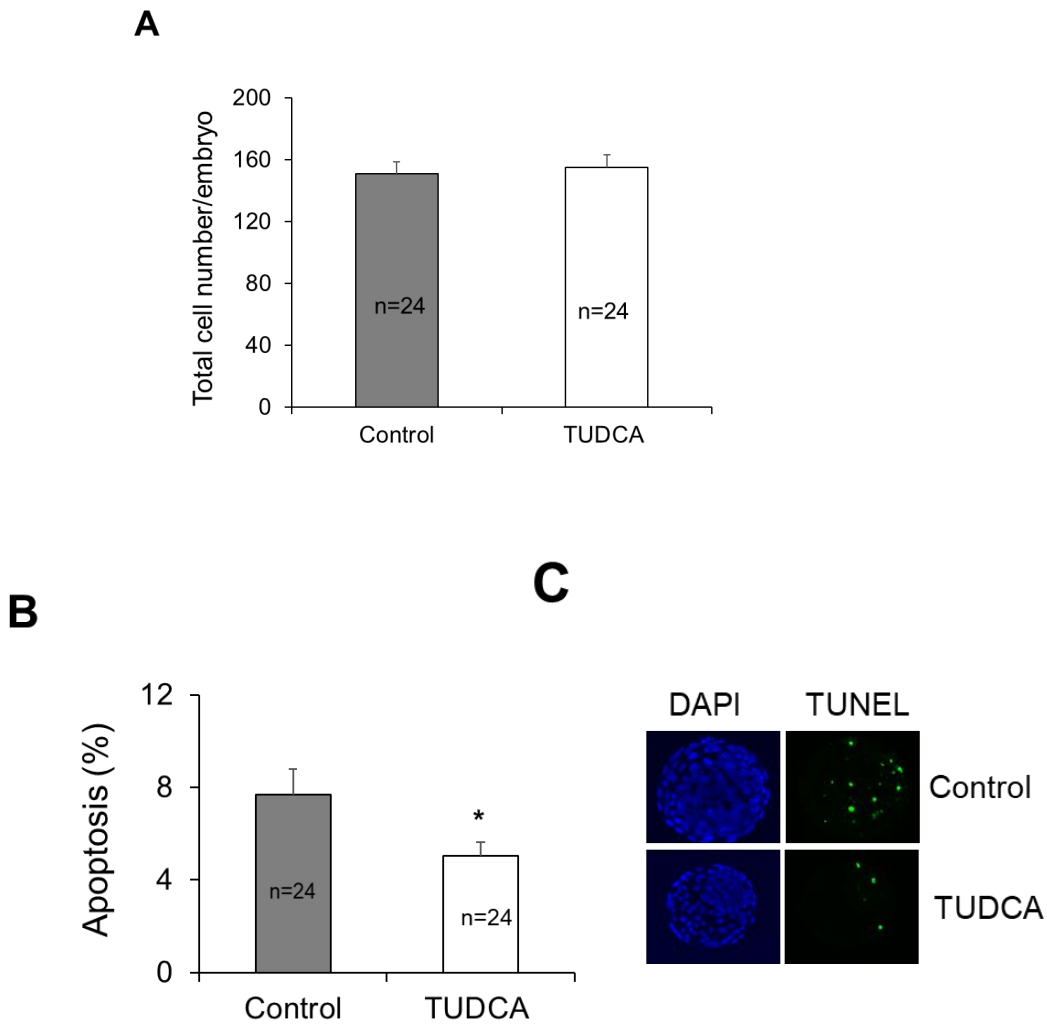

Figure 4.6 Effects of TUDCA during IVM on total cell number (A) and the percentage of apoptotic cells (B) in blastocysts. Bovine COCs were cultured in the presence or absence of TUDCA (100 μM) during IVM followed by *in vitro* fertilization and embryo culture for 8 days. (C) Representative images of Day 8 blastocysts applied for TUNEL staining from the control and TUDCA groups. TUNEL (green) and DAPI (blue) staining were used to detect apoptotic cells and DNA, respectively. The values are means ± SEM from five independent experiments. *: $P < 0.05$. TUDCA: tauroursodeoxycholic acid; IVM: *in vitro* maturation; DAPI: 4′,6-diamidino-2-phenylindole; TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

4.5 Discussion

The present study demonstrates that ER stress generally occurs and activates UPR signaling pathways in bovine COCs during IVM, and inhibition of ER stress via TUDCA interferes the action of ER stress triggering molecules and retrieve the developmental process of oocytes. For any chemical treatment, the timing and concentration of the chemical applied is crucial and should be optimized to maximize output (Kishigami, et al. 2006). Based on the working concentrations provided by the ER stress inhibitor TUDCA, different concentrations were examined for evaluation of their efficiency. In this study, 100 µM TUDCA significantly increased the meiotic maturation, COCs quality as evidenced by decrease in apoptotic cells and increase subsequent embryonic development after IVF. Until now, this is the first report to demonstrate that optimize concentration of TUDCA is protective in ER stress-induced impairments of oocyte maturation in bovine.

It has been shown that TUDCA can reduce ROS level and modulate mitochondrial turnover (Fonseca, et al. 2017, Rodrigues, et al. 2003). Taking this fact into consideration, this study evaluated the potential of TUDCA to abrogate ROS production and apoptosis. ROS generated by ER stress could affect mitochondria function and finally lead to apoptosis. TUDCA treatment significantly reduced the ROS content, apparently decreased the number of apoptotic cells, significantly downregulated the pro-apoptotic gene *BAX*, and upregulated the anti-apoptotic gene *BCL2* expression in matured COCs. These results indicate that TUDCA mediates cell death and reduces ROS production during maturation of bovine COCs. Moreover, the present study showed that the point at which TUDCA was applied greatly influenced the progress of COCs maturation. During IVM, COCs treated with TUDCA (100 µM) significantly decreased the ROS content compared to the control group (Fig. 4.1B), whereas denuded oocytes treated with TUDCA (100 µM) showed similar level

of ROS production in the control group (Fig. 4.1C), indicating that cumulus cells are more vulnerable to ER stress and diminishes the oocyte quality during maturation. The relief of ER stress by TUDCA treatment is a well-characterized, standard *in vitro* study model. Due to high antioxidant and anti-apoptotic properties, TUDCA can mitigates ROS generation to directly suppress oxidative stress and ER stress-induced apoptosis (Boyce, et al. 2005, Yoon, et al. 2014).

Furthermore, it also observed that 100 µM TUDCA is the optimal concentration for bovine COCs maturation. Maturation rate of oocytes was similar between control and 50 µM TUDCA groups, and rate was significantly low in comparison with the 100 µM TUDCA group. In contrast, maturation rate tended to decrease and evidently show many apoptotic cells, when TUDCA was administered at a higher (200 µM) concentration (Table 4.2 and Fig. 4.2A). Previous study has shown that high TUDCA concentrations exhibit toxicity to cultured cells/embryos (Jia, et al. 2018). This follow-up study also revealed that the optimal concentration of TUDCA for IVM was higher than that of IVC in bovine. These findings suggested that the TUDCA concentration used for treatment should be optimized depending on the developmental period.

The present study also investigated the effect of TUDCA on reducing ER stress via the regulation of UPR signaling protein/gene expression in matured COCs. As expected, the localization and amount of GRP78/BIP protein/mRNA expression significantly decreased in COCs treated with TUDCA, whereas these parameters were significantly increased in control group. GRP78/BIP, which is encoded by *HSPA5*, contributes to protein folding in the ER compartment, and higher expression of *GRP78/BIP* at the transcriptional level is a marker of cellular ER stress (Park, et al. 2017). The activation of *GRP78/BIP* regulates the

PERK-eIF2a pathway, which induces the transcription factors *ATF4* and *CHOP*, markers of apoptosis initiation (Wang, et al. 2013a). A recent study reported that the UPR signaling arm *PERK-ATF4* influences the expression of *IRE1α*, thereby elevating the ratio of *XBP1* mRNA splicing (Tsuru, et al. 2016). Taking these data into consideration, we reasoned that the increase in the ER chaperone GRP78/BIP expression at the protein or mRNA level activates the UPR signaling genes *PERK, ATF4, CHOP, IER1*, and *XBP1* in COCs during maturation. Importantly, TUDCA treatment significantly decreased the expression of these genes, indicating that the ER stress-induced UPR signaling arm *PERK-eIF2a-ATF4-CHOP* or *IER1-XBP1* is inhibited by TUDCA. These findings were also supported by the downregulation of the pro-apoptotic gene BAX in COCs treated with TUDCA, because this apoptotic molecule interacted directly with the cytosolic domain of IRE1 during excessive ER stress (Hetz, et al. 2006). However, there was no significant effect of TUDCA on *ATF6* gene expression, suggesting that protein translation was less affected. These results are supported by many studies implicating TUDCA as a chemical chaperone that can mitigate ER stress-induced UPR signaling (Uppala, et al. 2017).

Finally, this study demonstrated that inhibition of ER stress via TUDCA during IVM significantly ($P < 0.05$) improves the developmental rate and quality of embryos after IVF. Similar observation was found in porcine, where TUDCA promotes maturation of COCs and embryonic development by inhibiting ER stress (Zhang, et al. 2012). However, the remedial mechanism through which TUDCA exerts its effect on the maturation of oocytes and improves their further embryonic development is not entirely clear yet. One possible prediction here that the beneficial effect of TUDCA on embryo development after IVF may rely upon the oocyte quality obtained during IVM, which probably maintains the redox homeostasis and prevents oxidative stress after IVM. In the literature, TUDCA has been

widely reported as a strong ER stress inhibitor and to improve the quality and developmental competence of oocytes/embryos (Basar, et al. 2014, Song, et al. 2012, Yoon, et al. 2014, Zhang, et al. 2012).

Collectivey, these results show that ER stress during IVM is a crucial fact for the poor developmental competence of bovine COCs. Whereas, treatment of COCs with ER stress inhibitor TUDCA during IVM improves the bovine oocyte maturation and subsequent embryonic development by suppressing ER stress-induced UPR signaling pathways, ROS generation and apoptosis in COCs during maturation.

Chapter 5

Autophagy induction improves the developmental competence and quality of bovine embryos produced *in vitro*

5.1 Abstract

The aim of this study was to investigate whether the induction of autophagy during culture influences *in vitro* bovine embryo development and quality in the viewpoint of genes expression. Bovine embryos were culture in IVC medium supplemented with rapamycin and wortmannin, an autophagy-inducer and -inhibitor, respectively; and during culture, 2 and 4 cell-embryos were collected for the detection of autophagic activity. The results show that treatment of rapamycin significantly increased the expression of all autophagy-related genes (*LC3, ATG5, ATG7)* and protein (ratio of LC3-II/I) in 4 cell-embryos; while the expression of *mTOR* significantly decreased in both 2 or 4 cell-embryos compared to control and wortmannin. The activation of the autophagic pathway was further confirmed by a significant decreased of maternal genes *BMP15, GDF9* and *FST* in 4 cell-embryos treated with rapamycin. Rapamycin treatment during IVC significantly increased the cleavage and developmental rates, and impaired apoptosis at blastocyst stage; whereas these parameters were reversed by wortmannin. Consequently, when embryos treated with rapamycin for the first 3 days of IVC, embryonic development to the blastocyst stage significantly improved (49.9 ± 1.5%) compared to the control group (43.4 ± 1.0%); whereas, the treatment of wortmannin showed detrimental effects on blastocyst formation (17.0 ± 0.6%) than in the control. In addition, rapamycin significantly upregulated expression of development related genes *BCL2, MnSOD, SOX2, POU5F1, NANOG, PLAC8, IFN-tau,* and *GLUT5* in blastocysts than in the control. These results suggest that rapamycin plays a positive role on the developmental competence and quality of bovine embryos by increasing autophagy activity at early stage of development.

5.2 Introduction

In recent decades, the molecular mechanisms of autophagy and its role in reproductive system have been gone into and provided an insight that autophagy synchronized early embryogenesis, when induced by chemical compounds in mammalian species. Autophagy is ongoing at basal levels within cells, but is induced under unfavorable circumstances (Mizushima 2018). In mammalian reproduction and development, autophagy is implicated in the maintenance of primordial follicles, blastocyst cavitation, and fetal survival at birth (Lim and Song 2014). In addition, cold stress during vitrification and maternal diabetes induce autophagy in mammalian oocytes (Adastra, et al. 2011, Bang, et al. 2014). The activation of autophagy is important for turning over maternal proteins to newly synthesized embryonic ones during preimplantation embryonic development (Tsukamoto, et al. 2008a). It has been shown that induction of autophagy via rapamycin increased the expression of ATG5, an essential protein for autophagosome, and this ATG5 transgenic mice shows higher GSH concentration than wild-type mice (Pyo, et al. 2013); which might be contributed to improve embryonic developmental competence. Accumulating evidences suggest that the activation of autophagy is indispensable for embryogenesis, placentation, implantation as well as reprogramming in somatic cell nuclear transfer (Shen, et al. 2015).

Rapamycin is a natural product with potent antifungal and immunosuppressive activities. It inhibits Ser/Thr protein kinase and act as a foremost regulator of metabolism, cellular proliferation, stress responses, growth, and cell cycle progression (Laplante and Sabatini 2009). In the literature, rapamycin has been widely reported as a selective inhibitor of mammalian target of rapamycin (mTOR), (Choi, et al. 2014, Thoreen, et al. 2009) and to induce autophagy in oocyte maturation or embryo/cell culture (Lee, et al. 2016, Lee, et al. 2015, Shen, et al. 2015, Song, et al. 2014, Tsukamoto 2015). In contrast, wortmannin is a

fungal metabolite, cell-permeable that acts as a potent, selective and irreversible inhibitor of phosphatidylinositol 3-kinase (PI3K) and to inhibit autophagy (Arcaro and Wymann 1993, Blommaart, et al. 1997).

Despite, the function of autophagic activity have been well observed in mice embryogenesis, however role of autophagy in bovine embryogenesis is largely unknown. In addition, the evaluation of autophagic activity on embryo quality at the viewpoint of genes expression is still a key focus. Consequently, the aims of this study were to examine the level of autophagic activity in embryos at 2 and 4- cell stage and to investigate whether the induction of autophagy during culture influences *in vitro* bovine embryo development and quality in the terms of genes expression.

5.3 Materials and methods

5.3.1 *In vitro* embryo production

Good quality COCs were aspirated from bovine ovaries obtained from a local abattoir and *in vitro* embryo production was carried out based on the methods described in chapter 2 (see section 2.3.2 and 2.3.3).

5.3.2 Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining

It was carried out based on the procedures described in chapter 2 (see section 2.3.5).

5.3.3 Western blot analysis

Embryos (about 30 embryos at 2 or 4 cell stage per sample) were washed three times with PBS and lysed in 40 µL lysis buffer (Bolt LDS Sample Buffer, Thermo Fisher Scientific) and subsequently denatured at 70 °C for 10 min. The protein samples were separated by electrophoresis in Bolt™ 4–12% Bis-Tris Plus SDS polyacrylamide precast gels (Invitrogen) and transferred to PVDF membranes using an iBlot®2 Dry Blotting System (Thermo Fisher Scientific). The membranes were blocked by incubation in PVDF blocking reagent (Cat. No. B1140201, Toyobo) for 1 h and then washed three times with TBS-T at room temperature. Membranes were incubated with primary anti-LC3-I/II polyclonal antibody (1:1000; Cat. No. PM036, MBL, Nagoya, Japan) and anti-β-actin (1:5000; Cat. No. 010-27841, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) antibodies at 4 °C overnight. On the next day, after three washes with TBS-T, membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibodies (1:30000 dilution; Cat. No. NA931, GE Healthcare) at 37 °C for 1 h. Primary and secondary antibodies were diluted with immunoreaction enhancer (Can Get Signal; Toyobo).

Membranes were washed extensively with TBS-T before bound antibody detection using the ECL-Prime Western Blotting Detection system (GE Healthcare) as per the manufacturer's instructions. Immunoreactive signals were captured and analyzed utilizing image J software 1.55 (National Institutes of Health).

5.3.4 qPCR analysis

Total RNA (approximately 10 blastocysts or 15 embryos at 2 or 4 cell-stage per sample) was isolated using an RNeasy Micro Kit (QIAGEN) according to the manufacturer's instructions. Extracted RNAs were either used immediately for RT or stored at −80°C. The RT was performed using the SuperScript™ VILO™ Master Mix cDNA Synthesis Kit (Invitrogen) according to the manufacturer's instructions. The RT mix (20 µl) consisted of 12 µl of RNA, 4 µl of SuperScript™ VILO™ Master Mix, 4 µl of nuclease-free water. The RT reaction was performed with annealing of random primers (10 min at 25°C), cDNA synthesis (120 min at 42°C), and termination of the reaction (5 min at 85°C). Each sample was analyzed in duplicate using the SsoAdvanced™ universal SYBER® green supermix with CFX Connect (Bio-Rad). The real-time PCR mix (20 µl) consisted of 2 µl cDNA, 10 µl supermix, 6 µl nuclease-free water, and 1 µl each of forward and reverse primers for each gene. The program used for the amplification included a denaturing cycle (30 s at 95°C), 40 cycles of PCR (95°C for 5 s and 58°C for 5 s when used *RN18S1* or 95°C for 5 s and 60°C for 5 s when used *H2A*), and a melting curve program (60–95°C with a heating rate of 0.5°C/s, continuous fluorescence acquisition, and cooling to 30°C). The fold changes of target genes were evaluated by the ΔΔCT method using endogenous reference gene (*RN18S1*or *H2A*) expression. The PCR primers used to amplify each gene are listed in Table 5.1.

Table 5.1 List of primers used in PCR

Gene name	Primer sequence	Product size (bp)	Accession no.
BAX	F: agcgcatcggagatgaattg R: aaacatttcagccgccactc	110	NM_173894.1
BCL2	F: tggatgaccgagtacctgaa R: gagacagccaggagaaatcaaa	123	NM_001166486.1
MnSOD	F: gcttacagattgctgcttgt R: aaggtaataagcatgctccc	101	S67818.1
IFN-tau	F: tccatgagatgctccagcagt R: tgttggagcccagtgcaga	103	X65539
POU5F1	F: tgcagcaaattagccacatc R: aatcctcacgttgggagttg	123	NM_174580.2
PLAC8	F: cggtgttccagaggtttttcc R: aagatgccagtctgccagtca	163	NM_016619
NANOG	F: cccggtcaagaaacaaaaga R: tctggaaccaggtcttcacc	161	NM_001025344.1
SOX2	F: catgtcccagcactaccaga R: ctccatgctgtttcttgctg	161	NM_001105463.2
GLUT5	F: atctccgtgctgaagctgtt R: ttggacatcgtcctcattca	159	AF308830.1
BMP15	F: gcagccaagaggtagtgagg R: caatactgcctgcttgacga	194	NM_001031752.1
GDF9	F: ctcctcttgagcctctggtg R: acagccctctcttctggtca	243	NM_174681.2
FST	F: cagtgcctgtcacctgagaa R: cctctgccaaccttgaagtc	150	BF774514.1
LC3	F: ctacctgcatggggttcact R: atgcagcaggaagagcagat	225	NM_001001169.1
ATG5	F: ccactgccgtcattaaacct R: ttccactccctcgagctaaa	212	NM_001271737.1
ATG7	F: aaggaggccagtcacaaatg R: tcacgatccaagaagggaac	197	NM_001083795.2
mTOR	F: ccaccagcttctcacagaca R: cagctcctcactcaccatca	178	XM_001788228.1
H2A	F: acagctgtccagtgttggtg R: gcagaaatttggttggttgg	125	NM_174809
RN18S1	F: aaacggctaccacatccaagg R: gcggaaggatttaaagtggactc	138	DQ066896

5.3.5 Experimental designs

Experiment 1: Effects of rapamycin or wortmannin on expression of autophagy-related protein/genes

To examine the level of autophagic activity, embryos were cultured in IVC medium supplemented with 100 nM rapamycin or 10 μM wortmannin, an autophagy-inducer and -inhibitor, respectively, and collected 2 and 4 cell-embryos during culture. Then analyzed autophagy-related genes *LC3, ATG5, ATG7,* and autophagy inhibitory gene *mTOR* in 2 and 4 cell-embryos by qPCR. Next, the conversion of LC3-I to LC3-II, a keen of autophagy monitor, was measured in 2 and 4 cell-embryos by western blots.

Experiment 2: Effects of autophagy activation on maternal mRNA degradation

In this experiment, to confirm the activation of autophagy signaling pathway, three rapidly degradation maternal mRNA (*BMP15*, *GDF9* and *FST)* levels were detected in 2 or 4 cell-embryos obtained from control, rapamycin or wortmannin group by qPCR.

Experiment 3: Effects of autophagy induction on blastocyst outcomes and quality

To elucidate the beneficial effects of rapamycin induced autophagy, zygotes were cultured in the presence or absence of 100 nM rapamycin or 10 μM wortmannin until Day 8 and developmental competence was examined as the cleavage and blastocyst rates on Days 2 and 8, respectively. The total cell number and apoptotic status of the blastocysts at Day 8 were also assessed to investigate embryo quality by performing a TUNEL assay.

Experiment 4: Effects of autophagy induction on development-related gene expression

To explore the mechanism by which induction of autophagy enhances the developmental rate and quality, embryos were treated either with or without rapamycin (100 nM) during

IVC and blastocysts at Day 8 were analyzed for expression of apoptosis (*BAX* and *BCL2*) and development (*MnSOD, SOX2, POU5F1, NANOG, PLAC8, IFN-tau,* and *GLUT5*) related genes that are mainly induced during culture and essential for retaining embryo development and quality.

Experiment 5: Effects of timing of autophagy activation during IVC on developmental competence of embryos.

To confirm the timing of autophagy activation, the presumed zygotes were cultured in IVC medium in the presence or absence of rapamycin (100 nM) or wortmannin (10 μM) for first 3 days of IVC and subsequently cultured in medium without these agents until Day 8. The percent of embryos that cleaved was assessed on Day 2 and the percent of embryos that developed to the blastocyst stage was determined at Day 8 (day 0 represents the day of insemination).

5.3.6 Statistical Analysis

For each experiment, at least four independent replicates were performed unless specified otherwise. Data are presented as mean ± SEM. All statistical analyses were carried out using BellCurve for Excel version 3.20 (Social Survey Research Information). Differences between two groups were assessed by Student's t-test. Differences among three or more groups were examined using ANOVA followed by the Tukey-Kramer multiple comparison test. All percentage data were arcsine transformed prior to statistical analysis. A *P*-value < 0.05 or < 0.01 were considered statistically significant.

5.4 Results

5.4.1 Effects of rapamycin or wortmannin on autophagy-related protein/gene expression

To evaluate the level of autophagic activity, embryos were treated with rapamycin or wortmannin during IVC and analyzed the relative expression of autophagy-related genes *LC3, ATG5, ATG7,* and *mTOR* in 2 and 4 cell-embryos by using qPCR. The results show that induction of autophagy with rapamycin significantly upregulated all autophagy-inducing genes in 4 cell-embryos in comparison with the control and wortmannin groups ($P < 0.05$; $P < 0.01$; Fig. 5.1 A, B and C). On the other hand, expression of *mTOR*, a negative regulator of autophagy significantly decreased in both 2 or 4 cell-embryos treated by rapamycin than in the other groups (Fig. 5.1D; $P < 0.01$). Next, to investigate when autophagy actually occurs, conversion of LC3-I to LC3-II, the main indicator of autophagy induction was measured in 2 or 4 cell-embryos by western blotting. The treatment of rapamycin markedly increased the ratio of LC3-II/I in 4 cell-embryos; whereas wortmannin treatment significantly decreased the ratio of LC3-II/I in both 2 or 4 cell-embryos (Fig. 5.2; $P < 0.05$; $P < 0.01$).

5.4.2 Induction of autophagy influences the maternal mRNA degradation

To ensure the activation of autophagy and its role in the removal of obsolete maternal factors, three important maternal mRNA (*BMP15, GDF9* and *FST)* levels were detected in 2 or 4 cell-embryos treated with rapamycin or wortmannin. As shown in Figure 5.3, compared to control and wortmannin groups, rapamycin treatment dramatically decreased all maternal genes (*BMP15, GDF9* and *FST)* expression in 4 cell-embryos ($P < 0.05$; $P < 0.01$) and only *GDF9* in 2 cell-embryos ($P < 0.01$). Simultaneously, inhibition of autophagic activity with wortmannin significantly increased *GDF9* and *FST* genes in 4 cell-embryos.

5.4.3 Induction of autophagy enhances embryo developmental rate and quality

To define the role of autophagy in early embryonic development, embryos were cultured in the presence or absence of the rapamycin or wortmannin during IVC. The addition of rapamycin significantly improved cleavage and blastocyst developmental rate than in the control and wortmannin groups; whereas the rates were markedly decreased by wortmannin treatment compared with those in the other groups ($P < 0.05$; $P < 0.01$; Fig. 5.4A). Moreover, despite similar total cell number, rapamycin treatment showed a lower percentage of apoptotic cells in blastocysts than in the control and wortmannin groups ($P < 0.05$; $P < 0.01$; Fig. 5.4 C and D).

5.4.4 Induction of autophagy alters apoptosis-related mRNA expression

To examine the relationship between embryo autophagic activity and apoptosis, following culture, blastocysts at Day 8 obtained from the rapamycin and control groups were analyzed by using qPCR to detect the mRNA expression of *BAX* and *BCL2*. The results showed that the proapoptotic gene *BAX* has no significant difference in expression between groups; but the expression of the anti-apoptotic gene *BCL2* was significantly increased in blastocyst treated by rapamycin than in the control ($P < 0.05$; Fig. 5.5A).

5.4.5 Induction of autophagy activates developmentally related mRNA expression in blastocysts

To investigate the mechanism underlying autophagy enhances embryo development, the expression of developmentally related genes (those encoding *MnSOD, SOX2, POU5F1, NANOG, PLAC8, IFN-tau* and *GLUT5*) in blastocysts at Day 8 obtained from rapamycin and control groups were analyzed by using qPCR. Except *NANOG*, the relative expression

of all developmentally related gene significantly increased by rapamycin treatment compared with that observed in the control group ($P < 0.05$; $P < 0.01$; Fig. 5.5B).

5.4.6 Effects of timing of autophagy activation during of IVC on developmental competence of embryos.

Finally, to confirm the timing of autophagy activation, embryos were treated with rapamycin or wortmannin during the first 3 days of IVC and evaluated the developmental competence of embryos. As shown in Table 5.2, induction of autophagy via rapamycin supplementation significantly improved embryonic development to the blastocyst stage compared to the control group (49.9 ± 1.5% vs. 43.4 ± 1.0%; $P < 0.01$); whereas, the blastocyst formation rate was significantly decreased (17.0 ± 0.6%; $P < 0.01$) by wortmannin treatment compared with that in the control and rapamycin groups.

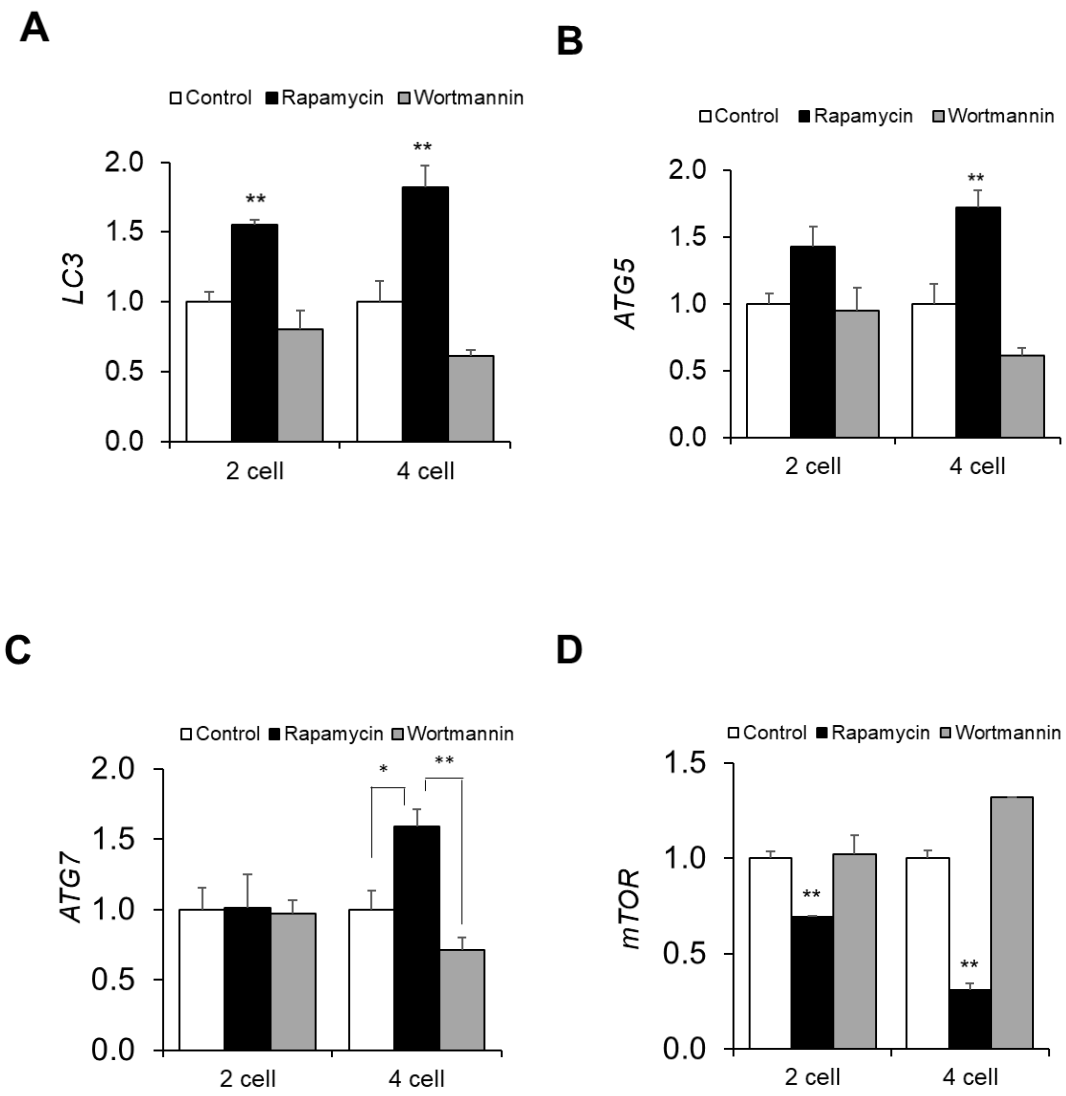

Figure 5.1 The relative expression of autophagy-related genes in 2 or 4 cell-embryos cultured in the presence or absence of rapamycin (100 nM) or wortmannin (10 µM), inducer and inhibitor of autophagy, respectively. Three replicates of 15 embryos each were assayed from each group by using qPCR. *H2A* was used as an internal standard. Values are the means ± SEM and marked with asterisks differ significantly (**$P < 0.01$; *$P < 0.05$). qPCR: quantitative polymerase chain reaction.

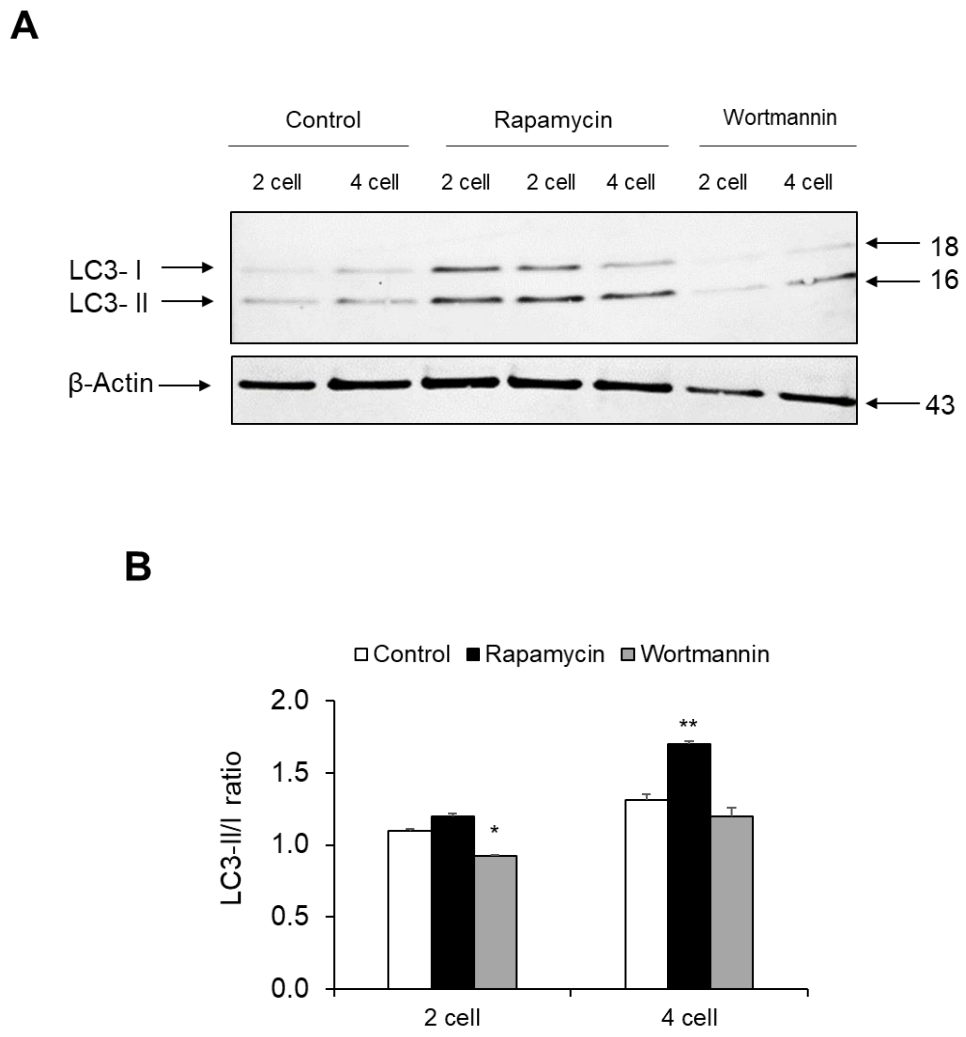

Figure 5.2 Western blotting assay for conversion of LC3-I (cytosolic) to LC3-II (autophagosome bound) in 2 or 4 cell-embryos cultured with or without rapamycin or wortmannin, inducer and inhibitor of autophagy, respectively. (A) Representative photograph is shown LC3-I (lane 1), LC3-II (lane 2), and β-actin (lane 3) levels as an internal control. (B) Quantification of the intensity of bands were expressed as the ratio of LC3-II to LC3-I, was carried out using Image J software. Values are means ± SEM from five replicates and marked with asterisks groups differ significantly (*$P < 0.05$; **$P < 0.01$).

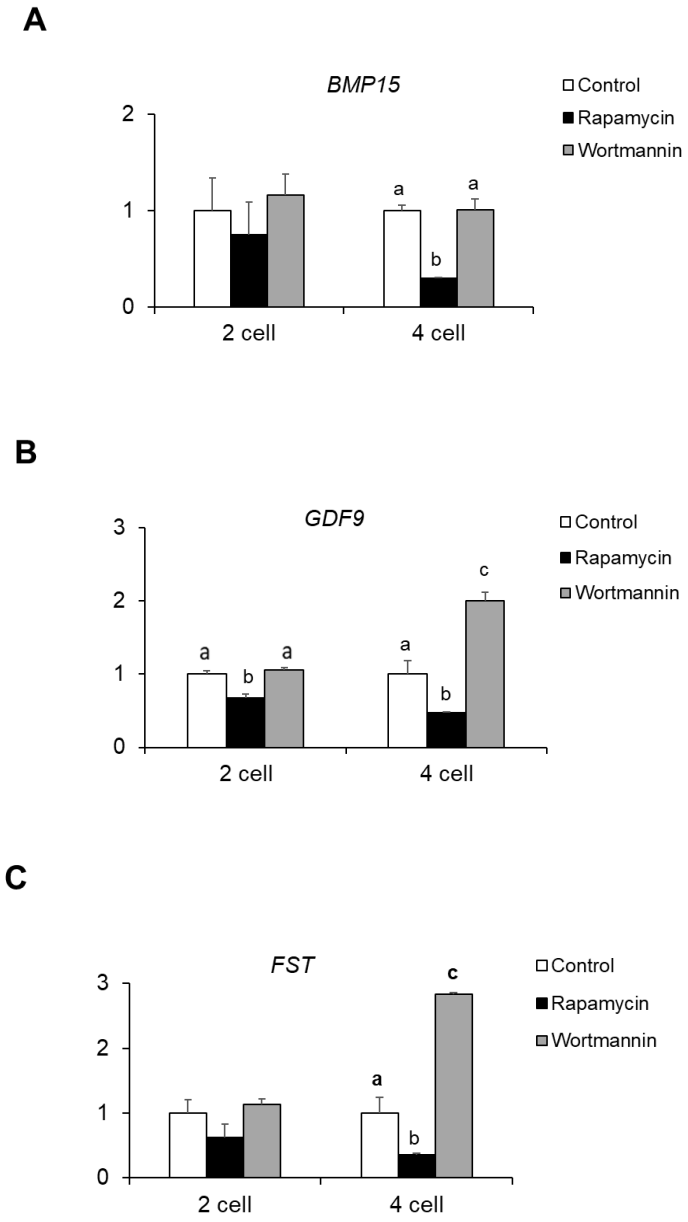

Figure 5.3 The relative expression of maternal genes *BMP15*, *GDF9* and *FST* in 2 or 4 cell-embryos cultured in the presence or absence of rapamycin or wortmannin, inducer and inhibitor of autophagy, respectively. Three replicates of 15 embryos each were assayed from each group by using qPCR. *H2A* was used as an internal standard. Values are the means ± SEM and different letters denote significant differences ($P < 0.05$; $P < 0.01$). qPCR: quantitative polymerase chain reaction.

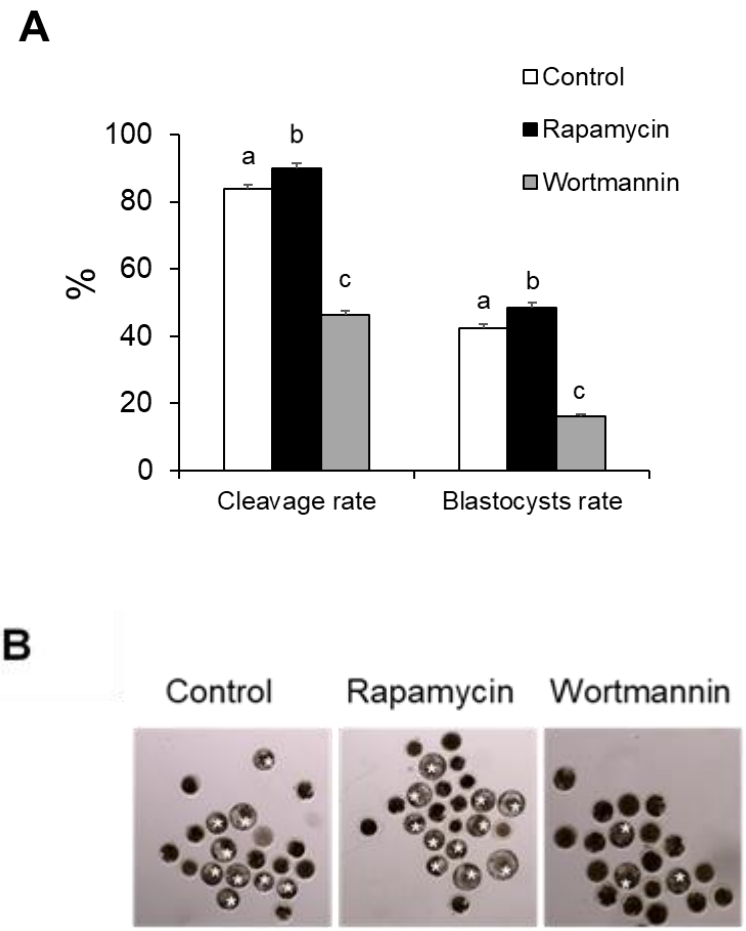

Figure 5.4 Effects of autophagy induction on (A) developmental rate, (C) total cell number, and (D) apoptosis in blastocysts following treatments with rapamycin and wortmannin. (B) Representative images of embryos at the blastocyst stage marked by a white star (*). (E) Representative images of Day 8 blastocysts and applied for TUNEL staining. TUNEL staining (green) was used to detect apoptotic cells and DAPI staining (blue) was used for DNA detection. The cleavage and blastocyst rates were examined on Days 2 and 8, respectively. The total number of embryos examined in control and rapamycin were over 44 and wortmannin was 18 from five replicates. Values are the mean ± SEM and different letters denote significant differences ($P < 0.05$; $P < 0.01$). DAPI: 4′,6-diamidino-2-phenylindole; TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

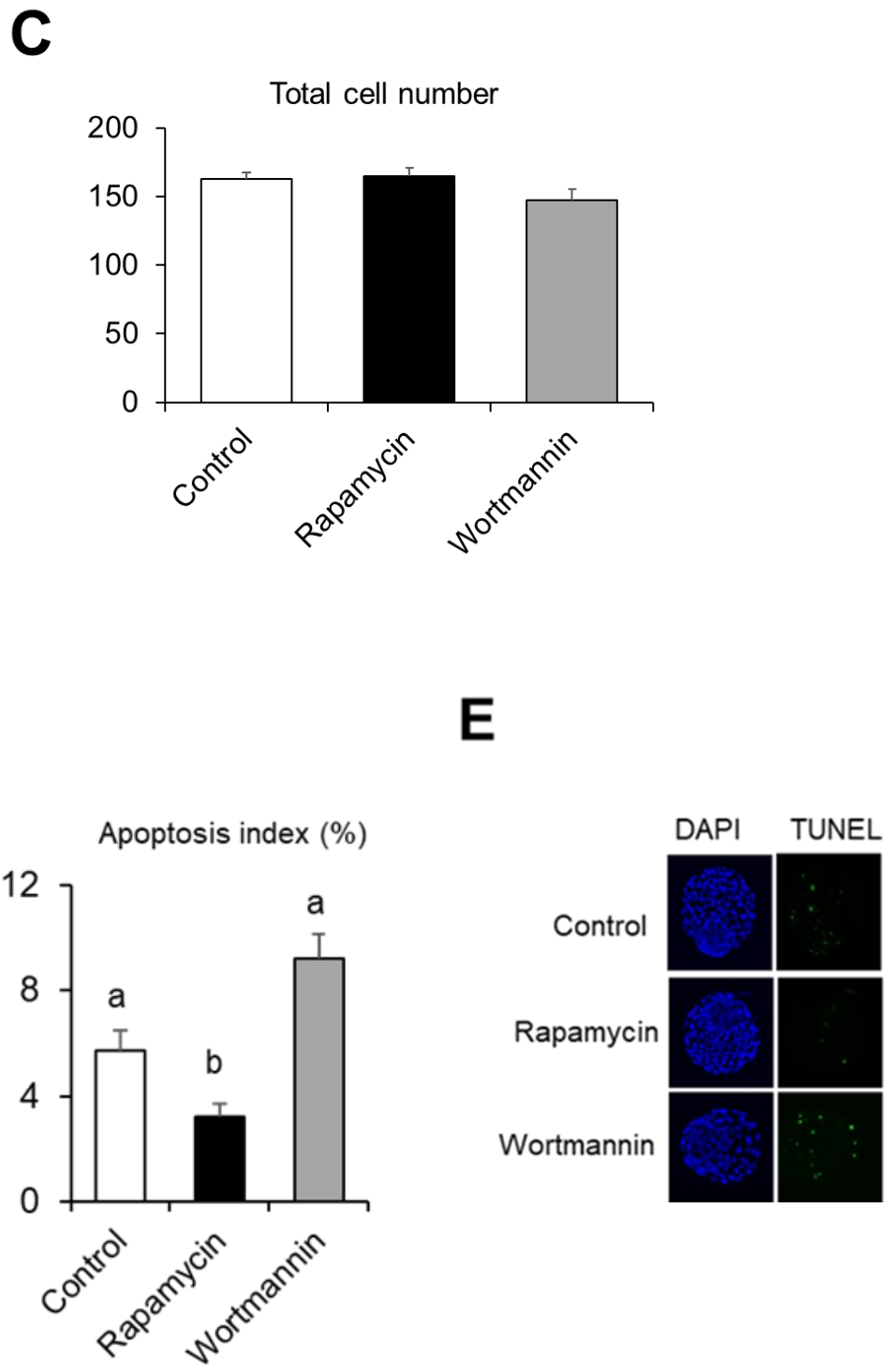

Figure 5.4 Continuation……

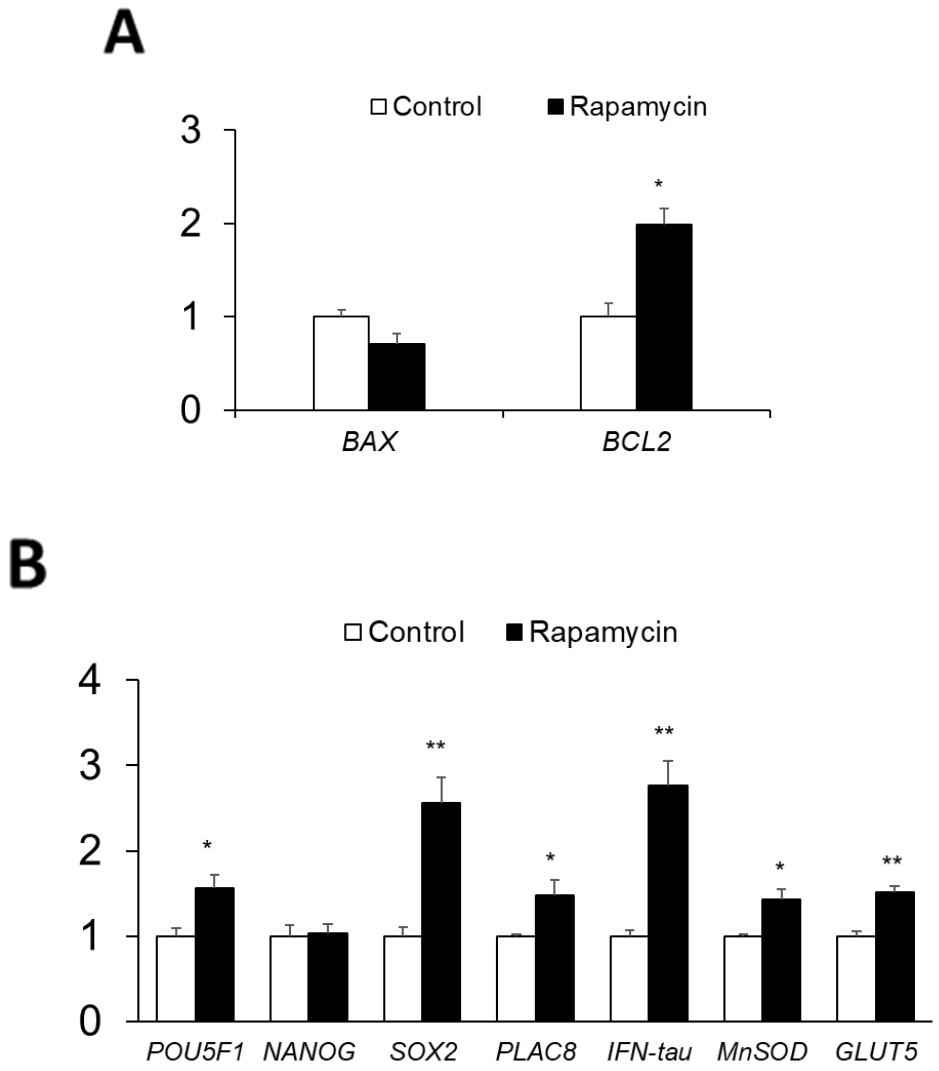

Figure 5.5 Effects of autophagy on the relative expression of apoptosis (A) and developmentally related (B) genes in blastocysts. Embryos were cultured in IVC medium either with or without rapamycin (100 nM) and mRNA expression in blastocysts at Day 8 was analyze by using qPCR. *RN18S1* was used as an internal standard. Values are the mean ± SEM from four independent replicates (about 15 embryos in each replicate) and marked with asterisks differ significantly (*$P < 0.05$; **$P < 0.01$). IVC: *in vitro* culture; qPCR: quantitative polymerase chain reaction.

Table 5.2 Effects of timing of autophagy during IVC on developmental competence of embryos.

Treatment	No. of embryos cultured	Percentage of embryos develop to			
		≥ 2 cells (%)	Total Blastocysts (%)	Expanded (%)	Hatched (%)
0 (Control)	176	84.7 ± 0.5[a]	43.4 ± 1.0[a]	23.5 ± 1.5	9.6 ± 0.4[a]
100 nM Rapamycin	181	91.1 ± 0.5[b]	49.9 ± 1.5[b]	26.5 ± 0.7	15.9 ± 1.2[b]
10 µM Wortmannin	170	47.0 ± 0.6[c]	17.0 ± 0.6[c]	0	0

Bovine embryos were cultured in IVC medium in the presence or absence of rapamycin (100 nM) or wortmannin (10 µM) autophagy-inducing and autophagy-inhibitory agents, respectively, for first 3 days of IVC and subsequently cultured in medium without the agents until Day 8. The percent of embryos that cleaved was assessed on day 2 and the percent of embryos that developed to the blastocyst stage was determined at day 8 (day 0 represents the day of insemination). Data are Mean ± SEM from four replicates. [a, b, c]Values with different superscripts within the same column are significantly different ($P < 0.05$; $P < 0.01$). IVC, *in vitro* culture.

5.5 Discussion

The present study demonstrates that modulation of the autophagy pathway using chemical modulators rapamycin (an autophagy inducer) during IVC influences the *in vitro* development and embryo quality in the viewpoint of genes expression. Based on literature, autophagic activation appears to be plateau from 2 to 4 cell-embryos in both mice and pig (Shen, et al. 2015, Tsukamoto, et al. 2008a, Xu, et al. 2012); considering that in the present study, bovine embryos at 2 and 4 cell-stage were collected for the detection of autophagic activity. The concentration of rapamycin (100 nM) or wortmannin (10 µM) used in the present study were determined from our previous work through a dose dependent experiment in bovine (unpublished). As a result, rapamycin treatment significantly increased all autophagy triggering molecules (*LC3, ATG5* and *ATG7*) in 4 cell-embryos; whereas only *LC3* gene was upregulated in 2 cell-embryos. A landmark study outlines, embryos derived from oocyte-specific *ATG5*-deficient mice die before implantation (Tsukamoto, et al. 2008b). In general, *ATG5* and *ATG7* mRNA play a role in the formation of an LC3-phosphatidylethanolamine complex, which is essential to the formation of autophagosomes (Nakashima, et al. 2017, Nath, et al. 2014). Thus, the expression of *ATG5* and *ATG7* mRNA are positively co-related with the level of *LC3 mRNA*, which is the main regulator of promoting the autophagic activity (Song, et al. 2014). In the process of autophagy, a cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine to form an LC3-phosphatidylehthanolamine conjugate (LC3-II), which is recruited to autophagosomal membranes (Kabeya, et al. 2000) and the activated membrane-bound derivative LC3-II protein expression is profoundly used to monitor autophagic activity. This is evident in the present study; rapamycin treatment markedly increased the conversion of LC3-I to LC3-II in 4 cell-embryos in comparison with the other groups. Moreover, upregulation of autophagy related protein/gene in this study further supported by the

downregulation of *mTOR* in both 2 or 4 cell-embryos treated with rapamycin, which has an inverse relationship with autophagy. These findings likely correspond to the regulation of autophagy with these modulators in embryos or cell type studies (Pan, et al. 2009, Ren, et al. 2009).

Autophagy is a dynamic bulk degradation system, so it is reasonable to postulate that maternal protein are degraded via autophagy in bovine embryos. To explore this probability, the three rapidly degrading mRNA (*BMP15, GDF9* and *FST)* levels in 2 or 4 cell-embryos were analyzed by using qPCR. Accordingly, induction of autophagy with rapamycin significantly decreased all maternal genes in 4 cell-embryos; while inhibition of autophagy with wortmannin maintained significantly high level of *BMP15* and *FST* gene in 4 cell-embryos. These results are supported by a previous study, in which porcine embryo treated with rapamycin decreases maternal mRNA level (*BMP15* and *CYCLIN B*) in both 2 or 4 cell-embryos (Xu, et al. 2012). Together, these findings indicate that the rapid degradation of maternal mRNA is, at least in part, triggered by autophagy. Furthermore, these observations are coincided with the significant upregulation of autophagy-related protein/gene expression in the present study. To sum up, this study suggests that rapamycin treatment persuades autophagy during bovine embryo development *in vitro,* which appear to be high in embryos at 4 cell stage compared to the 2 cell stage.

Next, it was examined whether the induction of autophagy influences embryo developmental competence in bovine. The results show that rapamycin treatment during IVC significantly increased the blastocyst developmental rate and decreased apoptosis; whereas wortmannin exhibited a significant hindrance to the development process. Consequently, rapamycin treatment for the first 3 days of IVC significantly improved

embryonic development to the blastocyst stage, while rate of blastocysts drastically decreased by wortmannin treatment during this time; suggesting that autophagy is activated at the early stage of embryonic development. These results suggest that the rapid intracellular metabolic turnover via rapamycin induced autophagy might be subsequently lead to high embryo development. In support of this, a study in mice recently reported that rapamycin accelerated active DNA methylation and removed the maternal mRNA, which are associated with the increase of embryonic development (Shen et al. 2015). Furthermore, the correlation between induction of autophagy and embryo development has been reported by many studies (Nakashima, et al. 2017, Song, et al. 2014, Tsukamoto, et al. 2014, Tsukamoto, et al. 2008a).

The molecular mechanism by which autophagy improves mammalian embryo development is not fully clear. On the other hand, the post-fertilization culture environment affects the developmental kinetics of bovine embryos and maintains blastocyst quality by inducing many genes essential for embryo development (Garcia, et al. 2015, Wang, et al. 2014). Thus, it has been hypothesized that the effectiveness of autophagy against embryo development might be embraced with its ability to activate developmentally related genes during culture. A set of genes possess several important physiological functions including: mitochondrial activity and detoxification of reactive oxygen species (*MnSOD*), pluripotency (*SOX2*, *POU5F1* and *NANOG*), placentation (*PLAC8*), implantation (*IFN-tau*), apoptosis (*BAX* and *BCL2*), transport and metabolism of fructose (*GLUT5*) in blastocysts were analyzed in the present study. The results showed that the expression of *MnSOD, SOX2, POU5F1, PLAC8, IFN-tau, BCL2* and *GLUT5* genes were significantly elevated in blastocysts obtained from the culture with rapamycin than in those from control. In mammalian cells, *MnSOD* is the only mitochondrial antioxidant enzyme that detoxifies the

free radical superoxide and is responsible for mitochondrial homeostasis (Candas and Li 2014, Sarsour, et al. 2012). Any stimuli during culture could induce *MnSOD* expression (Dhar and St Clair 2012); which is consistent with the present findings in which rapamycin increases *MnSOD* levels that assists embryo development.

Regarding the gene related with pluripotency (*SOX2*, *NANOG* and *POU5F1*), *SOX2* initiates autophagy and act as a critical transcriptional regulator in the oocyte-to-embryo transition (Wang, et al. 2013b). It is expressed throughout embryogenesis and is essential for maintaining the inner cell mass in blastocyst. *SOX2* form a regulatory core along with *POU5F1* and *NANOG*, for retaining pluripotency in embryos (Do and Scholer 2009, Keramari, et al. 2010). It was known that embryos treated with rapamycin greatly increased *POU5F1* mRNA level at the blastocyst stage in mice (Lee, et al. 2011). In this study, rapamycin also upregulates the *SOX2* and *POU5F1* genes level along with a positive effect on the blastocyst rate and quality. Collectively, these findings indicate that rapamycin induced autophagy could serve as a positive regulator of induced pluripotency.

The *PLAC8* and *IFN-tau* genes expression are widely used as a biomark of embryo quality. In ruminant, *IFN-tau* is known as a maternal pregnancy recognition gene. It is produced by trophoblasts and rapid upregulation of *IFN-tau* expression is associated with corpus luteum maintenance which is essential for pregnancy maintenance (Bazer, et al. 2008) and blastocyst cryotolerance (Rizos, et al. 2003). *PLAC8* placenta-specific gene is linked to cell invasion and has a potential role for placental development as well as for the fetus–maternal interface. Higher level of *PLAC8* in bovine blastocysts confirm the calf delivery (Ghanem, et al. 2011). In present study, the *PLAC8* and *IFN-tau* significantly increased by rapamycin

than in the control, which could specify the importance of autophagic activity for embryo apposition and pregnancy induction.

Indeed, the analysis of genes associated with apoptosis and the incidence of apoptosis in blastocyst are a reliable and predictive tool for the assessment of embryo quality and optimization of *in vitro* culture conditions. Moreover, embryo viability is associated with alterations in expression of cell death regulatory molecules (Jurisicova, et al. 2003). In the present study, regulation of autophagy with rapamycin significantly increased the anti-apoptotic gene *BCL2* expression, which can bind to *BECLIN1/ATG6* to inhibit *BECLIN 1*-mediated autophagy and autophagic cell death (Luo, et al. 2007). In addition, rapamycin treatment has a tend to decrease the pro-apoptotic gene *BAX* that induces apoptosis through release of cytochrome c from mitochondria to the cytoplasm (Gu, et al. 2015); which might underlie the higher incidence of apoptosis in control or wortmannin group (Fig. 5.4D). These findings were also accompanied by the reduction of apoptosis in embryos treated with rapamycin in the present study.

Furthermore, the present study also reports that rapamycin treatment significantly increased the expression of *GLUT5* gene, indicating that induction of autophagy accelerated the synthesis of nucleotides for the embryos during culture. Glucose is the main energy substrate for the embryo after the compaction stage and essential for the establishment of pregnancy (Harvey, et al. 2004). The upregulation of *GLUT5* gene in the present study is linked with the significant cleavage rate of embryos in rapamycin group, because *GLUT5* is highly expressed in fast-cleaving embryos (Gutierrez-Adan, et al. 2004). Growing body suggest that the metabolism and glucose uptake of the embryos was depend on embryo culture conditions (Harvey, et al. 2004, Morton, et al. 2007, Wrenzycki, et al. 2001). Nevertheless,

the expression of gene profile indicate the high quality of blastocysts obtained from the culture with rapamycin, which is linked with previous study demonstrated by (Tsukamoto, et al. 2014), where embryo with high autophagic activity during culture show high viability. These results suggest that rapamycin-assisted IVC leads to efficient autophagy activation in bovine embryogenesis, as apparent by synergistically an increase autophagy-induced protein/gene (*ATG8/LC3, ATG5* and *ATG7*) and a decrease autophagy-inhibitory gene *mTOR* along with rapid maternal mRNA degradation. More importantly, the present study discloses that extent of embryo autophagic activity during culture enhances blastocyst outcomes and embryo quality in the viewpoint of gene expression. Therefore, this study suggests that regulation of autophagic activity during culture is essential to improves *in vitro* embryo quality/viability.

Chapter 6

Summary and Conclusions

By all means, oxidative stress at the time of culture has a great impact on the developmental competence and quality of IVP oocytes/embryos. In addition, oxidative stress recently come to fore as an important component contributing to induce both ER stress and autophagy in mammalian reproductive systems. Evidences suggest that oxidative stress mediated ROS during culture impairs ER functions and triggers ER stress; beyond oxidative and ER stress accentuate each other in a positive manner, which interferes with mitochondrial function and activates pro-apoptotic signaling pathway; thereby leading to poor quality IVP embryos. Autophagy is an adaptive response that is induced in response to any stress and plays an important role in the homeostasis balances. Thus, controlling oxidative and ER stress and regulation of autophagy during *in vitro* culture is being increasingly recognized.

This dissertation analyzed the effects of oxidative and ER stress, and autophagy on bovine embryo development and quality under *in vitro* conditions. Currently, the silk protein sericin has been reported as a strong antioxidant that could enhance the developmental rate of IVP embryos under oxidative stress. Moreover, supplementation of sericin with IVC medium improves the quality of bovine embryos exposed to HS during culture. Based on the potentiality of sericin against oxidative and HS, it has been hypothesized that whether embryo produced by sericin supplementation medium has resistance to heat or cold, which is important for embryo transfer. Therefore, in this dissertation, to investigate the blastocysts viability in terms of thermotolerance, bovine embryos were cultured in IVC medium supplemented with or without 0.1% (w/v) sericin at 38.5°C until Day 7, and then exposed to HS (at 40.5°C for 6 h on Day 7) in the sericin-free medium followed by subsequently

cultured without sericin until Day 8 and analyzed the expression of developmentally related genes in blastocysts on Day 7 immediately after HS, and the rates of blastocysts development and apoptosis on Day 8. The results show that the quality was maintained in blastocysts obtained from the culture with sericin, as evidenced by the number of TUNEL-positive cells were significantly lower in blastocysts produced by sericin culture than in the HS control blastocysts. This result was supported by a significant decrease of *BAX*, a pro-apoptotic gene in blastocysts produced by sericin culture. Moreover, expression of gene *HSPA1A*, a marker of cell stress significantly decreases, while *IFN-tau*, maternal pregnancy recognition gene significantly increases in blastocysts produced by sericin culture; indicating that sericin improves the embryo quality in terms of thermotolerance. These findings suggest that supplementation of sericin with IVC medium can be a useful approach to improve IVP embryos viability in terms of thermotolerance in cattle.

Next, the relationship between ER stress and *in vitro* developmental kinetics of bovine embryos during culture, and its effect on embryo cryo-tolerance were examined. ER stress is a dysfunction in protein-folding capacity of ER that can be activated by many factors during culture and impairs the IVP embryo development and quality. To investigate this possibility, TUDCA was used to inhibit, while TM was used to induce ER stress in bovine embryos during culture *in vitro*. Accordingly, the result shows that activation of ER stress by TM treatment decreases the blastocyst developmental rate and increases the percentage of apoptotic cells; alternatively, the blastocyst developmental rate is increased, and the percentage of apoptotic cells are decreased in TUDCA group. Importantly, in the group treated with TM plus TUDCA, the developmental rate and the percentage of apoptotic cells in blastocysts were similar to that in the control group, indicating that TUDCA ameliorates the adverse effects of TM alone on embryo development and quality. In addition, significant

expression of ER stress-mediated UPR genes (*GRP78*, *ATF4*, *ATF6*, *IER1*, *sXBP1*, and *CHOP*) in control group confirms the existence ER stress during IVC. Because the expression of these genes drastically decreased by TUDCA treatment. Moreover, TUDCA decreases the ROS generation, expression of pro-apoptotic gene *BAX*; while it increases anti-apoptotic *BCL2* gene expression and glutathione levels. Further, ER stress inhibition during IVC enhances blastocyst cryo-tolerance as marked by a significantly increased hatching rate and decreased the number of apoptotic cells recorded at 48 h after a post-warming. However, the novelty of this study is that controlling ER stress via TUDCA (10 µM) during IVC increases embryos cryo-tolerance in cattle.

Based on these findings, a follow-up study conducted to investigate whether ER stress attenuation during IVM improves the oocyte developmental competence in bovine. For this purpose, bovine COCs were cultured in IVM medium containing TUDCA, an ER stress inhibitor. Consequently, administration of TUDCA in maturation medium promotes meiotic maturation and oocyte quality by regulating ER stress-induced UPR signaling pathways; thereby enhances the blastocyst formation rate and quality after IVF. These results suggest that ER stress and UPR signaling are intrinsic in oocyte maturation and that their proper function could be essential for meiotic maturation of oocyte and subsequent embryonic development *in vitro*.

Finally, the present study demonstrated that whether the modulation of embryo autophagic activity during IVC influences development and quality in bovine embryos. For this purpose, embryos were cultured in medium supplemented with rapamycin and wortmannin, an autophagy inducer and inhibitor, respectively. As expected, the rapamycin treatment accelerates the autophagic activity, as evidenced by significantly upregulates autophagy

triggering molecules (*LC3, ATG5, ATG7* genes and Lc3-II protein), suppresses *mTOR* expression and enhances rapid maternal mRNA degradation compared to control and wortmannin treatment. Further, induction of autophagy impairs apoptosis, increases blastocyst outcomes and activates many developmentally related genes (*BCL2, MnSOD, SOX2, POU5F1, NANOG, PLAC8, IFN-tau,* and *GLUT5*) in blastocysts. Based on these results, this study for the first time discloses that extent of embryo autophagic activity during culture involves with activation of many genes that are essential for blastocyst outcomes, quality and viability.

In conclusion, the findings of this dissertation focus on the regulation of oxidative and ER stress, and embryo autophagic activity during culture in IVP embryos and provide valuable new insight to develop culture medium and may be contribute to the production of high-quality bovine embryos *in vitro*. In addition, the data have important implications in the field of assisted reproduction and on future studies that investigate the mechanisms underlying the beneficial effects of sericin, TUDCA and rapamycin on development to term after the transfer of oocytes/embryos.

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