Endoplasmic reticulum stress attenuation promotes bovine oocyte maturation *in vitro*

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Abstract

We have previously reported that regulation of endoplasmic reticulum (ER) stress during *in vitro* culture acutely increases bovine embryo developmental rate and cryotolerance; these data indicate that ER stress is a critical factor reducing the quality of *in vitro*-produced embryos. In the current follow-up study, we examined whether ER stress attenuation during *in vitro* maturation influences meiotic maturation, oocyte quality, and subsequent embryonic development. Bovine cumulus oocyte complexes (COCs) derived from slaughterhouse ovaries were matured with or without tauroursodeoxycholic acid (TUDCA), a selective inhibitor of ER stress (0, 50, 100, and 200 μ M) for 22 h followed by *in vitro* fertilization, and zygotes were cultured for 8 days. Of the different doses of TUDCA, 100 μ M TUDCA significantly increased the maturation rate, and decreased reactive oxygen species in denuded oocytes, and appeared lower number of apoptotic cells in matured COCs. Subsequently, treatment of TUDCA (100 μ M) 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 μ M) during IVM significantly improved the blastocyst formation rate ($43.6 \pm 1.8\%$ vs $49.7 \pm 1.3\%$) and decreased the number of apoptotic cells ($7.7 \pm 1.1\%$ vs $5.03 \pm 0.6\%$) 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.

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Introduction

Mimicking *in vivo* conditions using *in vitro* (assisted) reproductive technology is a great challenge for researchers worldwide. Low-efficiency *in vitro* maturation (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% of oocytes reach the blastocyst 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 impaired cytoplasmic maturation, which is essential for the ultrastructural and functional modifications of oocytes allowing their fertilization and subsequent embryonic development (Rizos *et al.* 2002, Reader *et al.* 2017). Our recent study demonstrated that the use of *in vivo*-matured oocytes collected from superstimulated Japanese Black Cows enhances the quality of blastocysts produced *in vitro* compared to the *in vitro*-matured oocytes (Egashira *et al.* 2019). Therefore, it is necessary to understand the processes involved in the maturation of the cumulus oocyte complex (COC) *in vitro*.

Recent evidence suggests that stress to the endoplasmic reticulum (ER) is a major fact for poor developmental competence of oocyte maturation and embryo development *in vitro* (Lin *et al.* 2019). Oxidative stress is highly correlated with the function of ER protein-folding pathways; increased cytoplasmic ROS concentrations during maturation alter ER function and trigger ER stress (Sato *et al.* 2015, Burton *et al.* 2017). Furthermore, ER stress itself produces ROS and causes mitochondrial dysfunction (Malhotra & Kaufman 2007). This is important, because mitochondria activity has been linked to embryo development in both oocytes and pre-implanted embryos (Acton *et al.* 2004, Wu *et al.* 2010). However, the exact molecular mechanisms of ER stress in mammalian embryo development remain poorly understood, although it is generally accepted that the occurrence of ER stress can be detected by the expression of the unfolded protein response (UPR) pathway mediators, protein kinase-like ER kinase (PERK), activated transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1) (Tang & Yang 2015). The 78-kDa glucose-regulated protein (GRP78, also referred to as the immunoglobulin-binding protein

(BIP)) is an ER chaperone protein that is involved in many cellular processes, including the synthesis and folding new proteins, maintenance of ER calcium homeostasis, and serving as ER stress sensor (Wang *et al.* 2009). ER stress actively promotes the cell surface localization of GRP78/BIP, resulting in the dissociation of GRP78/BIP from its sensor protein in the ER membrane; furthermore, it activates the cytoprotective UPR (Kadowaki & Nishitoh 2013, Yang *et al.* 2016). However, the UPR fails when ER stress is excessive or persistent, and cellular apoptosis is induced through the activation of the CCAAT-enhancer-binding protein homologous protein (CHOP), Jun N-terminal kinase (JNK), and caspase pathway (Rasheva & Domingos 2009, Namba & Kodama 2015).

ER stress is involved in ovarian granulosa cell apoptosis (Lin *et al.* 2012), meiotic maturation (Yang *et al.* 2012), pre-implantation embryonic development (Yang *et al.* 2013), and embryo implantation and decidualization (Lan *et al.* 2013, Lin *et al.* 2014). Our previous work presents direct evidence that ER stress induction during *in vitro* culture interferes with the development of bovine embryos, and that this process can be restored by tauroursodeoxycholic acid (TUDCA), an ER stress inhibitor (Khatun *et al.* 2020). The occurrence of ER stress has also been confirmed during porcine oocyte maturation (Park *et al.* 2018). Therefore, it has been hypothesized that ER stress during IVM may be contributing to the poor developmental potency of bovine oocytes.

TUDCA is a nontoxic taurine conjugate 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). It is a safe cell-protective agent and is approved by the US Food and Drug Administration for the treatment of many diseases such as neurodegenerative disorders, inflammation, osteoarthritis, and diabetes (Vang *et al.* 2014). Both *in vitro* and *in vivo* studies have demonstrated that TUDCA functions as a chemical chaperone; it attenuates ER stress by preventing unfolded protein response dysfunction and stabilizing mitochondria (de Almeida *et al.* 2007, Chen *et al.* 2008, Vang *et al.* 2014). However, to date no investigations have examined the effects of TUDCA on bovine oocyte maturation.

Therefore, the aim of the present study was to determine whether the attenuation of ER stress during *in vitro* maturation improves the developmental competence of bovine oocytes. First, we investigated the most suitable concentration of TUDCA during IVM by examining the maturation rate, reactive oxygen species in matured denuded oocytes, and apoptosis levels in COCs. Next, to assess the mechanism by which TUDCA counteracts the detrimental effects of ER stress, the localization and amount of the ER stress-inducible GRP78/BIP protein expression in matured COCs were detected by immunohistochemistry and

western blot analysis, respectively. In addition, real-time quantitative PCR (RT-qPCR) was performed to analyze the expression of genes related to ER stress (GRP78/BIP, PERK, IER1, ATF6, ATF4, XBP1, and CHOP) and apoptosis (CASPASE 3, BAX, and BCL2) in matured COCs. Finally, we explored whether the inhibition of ER stress augments the developmental competence of oocytes by testing the effects of TUDCA supplementation during IVM on subsequent embryonic development *in vitro*.

Materials and methods

Chemicals

All chemicals were purchased from Sigma-Aldrich unless stated otherwise.

Oocyte collection and IVM

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 (Seika Pharma, Tokyo, Japan). 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 (Thermo Fisher Scientific) containing 5% (v/v) fetal bovine serum (FBS, Thermo Fisher Scientific). A group of 50 COCs were matured in a four-well multidish (Nunc, Roskilde, Denmark) containing 500 µL TCM-199 supplemented with 5% FBS, follicle-stimulating hormone (0.02 IU/mL; Kyoritsu Seiyaku, Tokyo, Japan), and gentamicin (10 µg/mL, Nacalai Tesque, Kyoto, Japan) covered with liquid paraffin (Nacalai Tesque) at 38.5°C for 22 h in a humidified atmosphere of 5% CO₂ in air.

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 Dulbecco's PBS (DPBS, Sigma) containing 0.5 mg/mL polyvinylpyrrolidone (PVP; Nacalai Tesque) 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).

Culture treatment

To modulate ER stress, COCs were matured in IVM medium with the ER stress inhibitor TUDCA (sodium salt; Calbiochem). 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 weeks. Next, the stock solution was diluted in IVM medium to obtain 50-, 100-, and 200-µM TUDCA solutions.

TUNEL staining

A TUNEL assay kit (in situ cell death detection kit; Roche) was used to evaluate the presence of apoptotic cells in matured COCs. Briefly, COCs were fixed in 4% (w/v) paraformaldehyde solution (pH 7.4) for 40 min, rinsed three times with 0.05% (w/v) PVP-PBS, and then permeabilized with 0.05% (w/v) PVP-PBS containing 0.5% TritonX-100 for 20 min followed by three washes with 0.05% (w/v) 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, COCs were washed three times with 0.05% (w/v) 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 under a fluorescence microscope (EVOS® FL, Thermo Fisher Scientific).

Assessment of oxidative stress

Oxidative stress as assessed by intracellular reactive oxygen species (ROS) in matured denuded oocytes, which were detected by the fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate (DCHFDA; Molecular Probes) as a green fluorescence signal. At least ten denuded oocytes from each treatment group were incubated in PVP-PBS containing 10 µM DCHFDA for 15 min at 38.5°C in a humidified atmosphere of 5% CO₂ in air followed by two washes with PVP-PBS. The oocytes 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 image was quantified using ImageJ software (version 1.55; National Institutes of Health) by counting the number of pixels after color inversion.

RT-qPCR analysis

RNA extraction and RT-qPCR were carried out according to the procedures described by [Khatun et al. \(2018\)](#). Briefly, total RNA (40 COCs per sample) was isolated using a RNeasy Micro Kit (QIAGEN) 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 (Invitrogen) 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 (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 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 $\Delta\Delta CT$ method using endogenous reference gene (RN18S1) expression. The PCR primers utilized to amplify each gene are listed in [Table 1](#).

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. 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) antibody at 4°C overnight, washed three times with 0.1% (w/v) PVP-PBS containing 0.1% (v/v) Tween-20 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 followed by washed three times with 0.1% (w/v) PVP-PBS containing 0.1% (v/v) Tween-20 for 10 min. Primary and secondary antibodies were diluted with Can Get Signal® Immunostain Immunoreaction Enhancer Solution A (TOYOBO, NKB-501). COCs were mounted on slides using mounting medium with DAPI and examined under a confocal laser microscopy (C1, Nikon, Excitation: 488 nm, Emission: 515 nm).

Western blot analysis

Matured 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™ 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 (B1140201; Toyobo, Osaka, Japan) for 1 h and then washed three times with 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, 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 NIH image software 1.55 (National Institutes of Health).

Table 1 Primer information.

Gene name	Primer sequence (5'–3')		Product size (bp)
	Forward	Reverse	
BAX	CTACTTTGCCAGCAAAGTGG	TCCCAAAGTAGGAGAGGA	158
CASPASE 3	CCCAAGTGTGACCACTGAAC	CCATTAGGCCACACTCACTG	169
PERK	GGCTGAAAAGATGACAGCACA	AGAACTGGCTCTCGGATGAA	195
GRP78/BIP	TGCAGCAGGACATCAAGTTC	TTTGTTTGCCACCTCCAAC	91
XPB1	TAGCAGCTCAGACTGCCAGA	ATCCCCCTTGGTCTCTGCTT	157
BCL2	TGGATGACCGAGTACCTGAA	GAGACAGCCAGGAGAAATCAAA	123
ATF6	TGAACTTCGAGGATGGGTTC	GAATTTGAGCCCTGTTCCAG	117
ATF4	GGCCAAGCACTTCAAACATC	AAGCATCCTCCTTGCTGTTG	110
CHOP	GCACCAAGCATGAACAGTTG	ATCGATGGTGGTTGGGTATG	116
IRE1	CCGAAGTTCAGATGGCATTC	TCTGCAAAGGCTGATGACAG	108
RN18S1	AAACGGCTACCACATCCAAGG	GCGGAAGGATTTAAAGTGGACTC	138
ACTB	ACGTCGCCTTGGACTTCGAGCAGG	GCTGGAAGGTGGACAGGGAGGCCAGGA	405

In vitro embryo production

In vitro fertilization (IVF) and embryo culture were carried out according to a previously described procedure (Yamanaka et al. 2018). Briefly, 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 (GE Healthcare). After removing the supernatant, the pellet was diluted with IVF100 solution (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 IVM, COCs were washed three times with IVF100, and IVF was performed at 38.5°C in 5% CO₂ 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 containing 5% 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% O₂ and 5% CO₂ and balanced with N₂ through Day 8 (Day 0 represented the day of insemination).

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% CO₂ in air and investigated the maturation rate of oocytes by acetic-orcein staining, and the intracellular ROS content by DCHFDA fluorescence.

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, RT-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, we analyzed the ER stress-associated genes (those encoding GRP78/BIP, PERK, IER1, ATF4, ATF6, CHOP, and XPB1) expression by RT-qPCR in COCs matured with or without TUDCA (100 µM).

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. 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.

Statistical analysis

Each trial of experiment run was accompanied by parallel control and same cultural conditions. For each experiment, at least three independent replicates were performed unless specified otherwise. Data are presented as mean ± S.E.M. 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 $P < 0.01$.

Results

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

The dose-dependent treatment of IVM culture with TUDCA suggested 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 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. 1). However, the percentage of MII-stage oocytes (Table 2) and the relative fluorescence intensity of the ROS content (Fig. 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 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. 2A). Furthermore, RT-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. 2C and D). However, no significant change was observed for *CASPASE 3* expression among the groups.

Effects of TUDCA on GRP78/BIP protein expression in COCs

As shown in Fig. 3A, both the number of GRP78/BIP protein aggregates and the fluorescence intensity were appeared higher in cumulus cells but not in oocyte under the control group, whereas COCs treated with TUDCA during maturation evidenced lower expression of GRP78/BIP in cumulus cells. 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 in comparison with the control group (Fig. 3B and C).

Effects of TUDCA on ER stress-associated genes expression in COCs

The RT-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*, *XBPI1*, and *CHOP*) in COCs in comparison with the control group ($P < 0.01$; $P < 0.05$; Fig. 4). However, TUDCA treatment had no significant effect on *ATF6* gene expression.

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

To determine the developmental competence of bovine COCs after inhibition of ER stress during IVM, 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 ($43.6 \pm 1.8\%$ vs $49.7 \pm 1.3\%$; $P < 0.05$; Fig. 5A) and decreased the number of TUNEL-positive cells in blastocysts ($7.7 \pm 1.1\%$ vs $5.03 \pm 0.6\%$; $P < 0.05$; Fig. 5D). However, no significant differences were found in the total cell number and embryo cleavage rate between the groups.

Discussion

The present study demonstrated that ER stress generally occurs in bovine COCs during IVM and activates the

Table 2 Effects of TUDCA supplementation during *in vitro* maturation on bovine oocytes.

TUDCA (μM)	No. of oocytes examined	Meiosis development (%)			
		GV	GVBD	MI	MI
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.

^{a,b}Values with different superscripts within the same column are significantly different ($P < 0.01$).

GV, germinal vesicle; GVBD, germinal vesicle break down; MI, metaphase I; MII, metaphase II; TUDCA, tauroursodeoxycholic acid. Data are mean $\pm$ S.E.M. from seven replicates.

Figure 1 Effects of TUDCA on intracellular ROS generation in oocytes during maturation. (A) Quantification of the relative ROS levels in denuded oocytes from the four different groups. (B) Representative images of ROS fluorescence (green) in denuded oocytes. Scale bar: 400 μm. Data are means $\pm$ S.E.M. from four replications. ** denotes significant differences ($P < 0.01$). TUDCA, tauroursodeoxycholic acid; ROS, reactive oxygen species.

UPR signaling protein/gene pathways. Furthermore, ER stress inhibition via TUDCA interferes with the effects of ER stress-triggered molecules and retrieves the developmental processes of oocytes. For any chemical treatment, the timing and concentration of the chemical applied is crucial and should be optimized to maximize the output (Kishigami *et al.* 2006). Different working concentrations of the ER stress inhibitor TUDCA were examined to evaluate their efficiency. We found that 100 μM TUDCA significantly increased meiotic maturation and COC quality as evidenced by decreased apoptotic cell number and increased subsequent embryonic development after IVF. To the best of our knowledge, this is the first report to demonstrate that optimized TUDCA concentration is protective in ER stress-induced impairment of bovine oocyte maturation.

The ER is a major intracellular organelle for the synthesis of transmembrane proteins and lipids and is involved in the maintenance of the intracellular calcium homeostasis (Yoshida 2007). A balance between ER protein synthesis and folding machinery is essential for proper protein folding. However, under *in vitro* conditions many stressors alter the ER function or intracellular homeostasis, leading to the accumulation of misfolded and unfolded proteins, a condition known as ER stress (Kaufman 1999). The literature has disclosed that ER stress and oxidative stress accentuate each other in a positive manner, which increases the ROS production

Figure 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. Scale bar: 400 μm. (B, C and D) The relative expression of CASPASES 3, BAX and BCL2 in COCs obtained from control and 100 μM TUDCA group measure by RT-qPCR. Values are means $\pm$ S.E.M. from four independent experiments. * $P < 0.05$; ** $P < 0.01$. TUDCA, tauroursodeoxycholic acid; COCs, cumulus oocyte complexes; RT-qPCR: real-time quantitative polymerase chain reaction; DAPI, 4',6-diamidino-2-phenylindole; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

and interferes with mitochondrial function and activates pro-apoptotic signaling pathways (Malhotra & Kaufman 2007, Yuzefovych *et al.* 2013, Yoon *et al.* 2014a). It has been shown that TUDCA can reduce ROS level and modulate mitochondrial turnover (Rodrigues *et al.* 2003, Fonseca *et al.* 2017). Taking this fact into consideration, we 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. 1), whereas denuded oocytes treated with TUDCA (100 μM) showed similar level of ROS production in the control group (Supplementary Fig. 1, see section on

Figure 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 $\pm$ S.E.M. and marked with asterisks groups differ significantly (** $P < 0.01$). COCs, cumulus cell complexes; TUDCA, tauroursodeoxycholic acid.

supplementary materials given at the end of this article), indicating that cumulus cells are more vulnerable to ER stress and diminishes the oocyte quality during maturation. These findings are supported by an elegant work, in which palmitic acids are known to induce

Figure 4 Effects of TUDCA on the relative expression of endoplasmic reticulum (ER) stress-associated genes (GRP78, PERK, ATF4, ATF6, IER1, XBP1, and CHOP) in COCs measured by RT-qPCR (three replicates) after treated either with or without TUDCA (100 μ M) during maturation. Values are means $\pm$ S.E.M. and marked with asterisks groups differ significantly (* $P < 0.05$; ** $P < 0.01$). COCs, cumulus cell complexes; TUDCA, tauroursodeoxycholic acid; RT-qPCR, real-time quantitative polymerase chain reaction.

Figure 5 Effects of TUDCA during IVM on the developmental competence of COCs. Bovine COCs were cultured in the presence or absence of TUDCA (100 μ M) 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 (*). (C) Total cell number and (D) percentage of apoptotic cells in blastocysts. (E) 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 total number of zygotes cultured under control and with TUDCA was 161 and 165, respectively. The values are means $\pm$ S.E.M. from five independent experiments. * $P < 0.05$. Scale bar: 400 μ m. TUDCA: tauroursodeoxycholic acid; IVM: in vitro maturation; COCs, cumulus cell complexes; DAPI: 4',6-diamidino-2-phenylindole; TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

ER stress in mouse COCs during maturation, impair mitochondrial activity and leading to compromise oocyte developmental competence that was reversed by salubrinal, an ER stress inhibitor (Wu *et al.* 2012).

Furthermore, we also observed that 100 μ M TUDCA is the optimal concentration for bovine COCs maturation. Maturation rate of oocytes was similar between control and 50 μ M TUDCA groups and rate was significantly low in comparison with the 100 μ M TUDCA group; indicating that this concentration was not enough for suppressing ER stress during culture. In contrast, maturation rate tended to decrease and evidently show many apoptotic cells, when TUDCA was administered at a higher (200 μ M) concentration (Fig. 2A and Table 2). Previous studies have shown that high TUDCA concentrations exhibit toxicity to cultured cells/embryos (Jia *et al.* 2018, Khatun *et al.* 2020). This follow-up study also revealed that the optimal concentration of TUDCA for IVM was higher than that of IVC in bovine (Khatun *et al.* 2020). These findings suggested that the TUDCA

concentration used for treatment should be optimized depending on the developmental period.

The relief of ER stress by TUDCA treatment is a well characterized, standard *in vitro* study model. Due to its high antioxidant potential, TUDCA prevents ER calcium depletion (Xie *et al.* 2002, Boyce *et al.* 2005), influences Akt-mediated MnSOD activation (Yun *et al.* 2018), and mitigates ROS generation to directly suppress oxidative stress (Yoon *et al.* 2014b). Moreover, TUDCA's anti-apoptotic properties have been reported by many studies. TUDCA prevents BAX translocation and interrupts the classical apoptosis pathway by modulating mitochondrial membrane perturbation. As a therapeutic agent, TUDCA has been effectively used to improve clinical symptoms and biochemical parameters in several liver diseases without exhibiting major side effects (Lebensztejn 2000, Nousia-Arvanitakis *et al.* 2001, Vandewynckel *et al.* 2015). These data supported our observation that TUDCA improves oocyte quality during maturation. In addition, here we speculate that TUDCA enhances the interaction between the oocyte and cumulus cells by reducing the incidence of apoptosis in a manner similar to that described previously (Rodrigues *et al.* 2003).

In the present study, we 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. In addition, immunohistochemical analysis showed that GRP78/BIP protein appeared higher in cumulus cells but not in oocytes under control group, which is coincided by the ROS level in denuded oocytes after maturation in the present study; clarify that cumulus cells are mainly exposed by ER stress. 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-eIF2 α pathway, which induces the transcription factors *ATF4* and *CHOP*, markers of apoptosis initiation (Wang *et al.* 2013). A recent study reported that the UPR signaling arm *PERK-ATF4* influences the expression of *IRE1 α* , 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-eIF2 α -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, we did not detect any 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, we 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. We speculate 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. We also used salubrinal as another ER stress inhibitor and found that salubrinal (400 nM) supplementation in IVM has no significant effect on the rate of oocyte maturation or subsequent embryonic development (Supplementary Table 1), which is similar to result reported by a previous study showing that treatment of salubrinal during IVM has no significant effect on oocyte maturation or embryonic development under normal conditions, although it attenuates nonesterified fatty acids (NEFAs) induced-ER stress in bovine COCs as indicated by increased subsequent embryonic development *in vitro* (Sutton-McDowall *et al.* 2016). Collectively, these results confirm that salubrinal is not as effective as TUDCA in the regulation of ER stress during maturation of bovine COCs. Interestingly, a comparative study demonstrated by Park *et al.* (2019) clearly show that the efficacy of TUDCA against ER stress is higher than salubrinal due to their different structure and mode of action. Salubrinal appears to be cytoprotective during ER stress by maintaining eukaryotic translation initiation factor 2 α (eIF2 α) phosphorylation and to reduce global protein synthesis (Boyce *et al.* 2005). Therefore, salubrinal is an eIF2 α -specific ER stress inhibitor. Whereas TUDCA is a hydrophilic bile acid and acts as bile secretagogues and immunomodulators that can stabilize the ER stress-induced UPR signaling pathway (Zhang *et al.* 2012, Vandewynckel *et al.* 2015). This high protective property of TUDCA may also lie in the presence of a taurine group, which enhances glucose metabolism by stimulating mitochondrial metabolic functions (Nunes *et al.* 2012). 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 (Song *et al.* 2012, Basar *et al.* 2014, Yoon *et al.* 2014b, Khatun *et al.* 2020).

In conclusion, our 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 via regulation of UPR signaling pathways, ROS generation and apoptosis in COCs during maturation.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-19-0492>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

H K designed and performed the experiments, analyzed data, and wrote the manuscript; Y W, T K and H T participated in the conception of the study design and revised the manuscript; K Y conceived the study design, participated in data analysis and interpretation, and revised the manuscript.

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