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Excessive expression and activity of cathepsin B in sheep cumulus cells compromises oocyte developmental competence

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ABSTRACT

In vitro developmental competence of sheep oocytes is still relatively low. In this study, we investigated cathepsin B activity in sheep cumulus-oocyte complexes, the expression of cathepsin B and apoptosis rates in sheep cumulus cells, and the correlations between these indicies and *in vitro* developmental competence of sheep oocytes. The present result suggested that the active form of cathepsin B in cumulus-oocyte complexes was predominantly present in cumulus cells rather than oocytes. Cathepsin B activity in cumulus-oocyte complexes was strongly inhibited by cathepsin inhibitor E-64. RT-PCR analysis showed that cathepsin B expression in cumulus cells decreased during *in vitro* maturation. TUNEL staining and flow cytometry of cumulus cells showed that the apoptosis rate increased after *in vitro* maturation, and treatment with E-64 reduced both cathepsin B expression and apoptosis levels. Furthermore, E-64 treatment increased blastocyst rates of sheep.

1. Introduction

In vitro embryo production (IVEP) is an assisted reproductive technology aimed at increasing the genetic diffusion of females. Nonetheless, there has been less research on IVEP in small ruminants such as sheep, compared with other livestock species. However, in recent years, there has been significant research in sheep embryo studies because of the rising importance of these animals in economically fast developing countries such as China and India and also owing to the growing interest in sheep. Although the technology of IVEP in sheep has been improved over time, the efficiency of IVEP is still lower than that of other livestock species.

The oocyte and surrounding cumulus cells are metabolically coupled through an extensive network of gap junctions (Albertini et al., 2001; Depalo et al., 2003; Eppig, 1991), sharing bidirectional communications of nutrients and molecule signals that regulate each other. Cumulus cells play an important role in nucleus and cytoplasm maturation of oocytes (Tanghe et al., 2002) and protect oocytes against oxidative stress-induced apoptosis (Tatemoto et al., 2000; Wongsrikeao et al., 2005).

Apoptosis, one kind of programmed cell death, is distinct from accidental cell death that is detrimental to an organism's health, and instead plays beneficial biological roles. However, excessive apoptosis can also become harmful and could lead to pathological consequences (Thompson, 1995). Apoptosis was found to play a critical role in the harmful effect of oocyte maturation (Roth and Hansen, 2004, 2005; Yuan et al., 2008; Soto and Smith, 2009). The apoptosis rate of cumulus cells surrounding oocytes matured *in vitro* is higher than that of cells matured *in vivo* (Salhab et al., 2013), and the apoptosis rate of cumulus cells was reported to be negatively related to developmental competence of bovine oocytes matured *in vitro* (Ruvolo et al., 2007; Yuan et al., 2005).

Cathepsin B (CTSB) plays an important role in mediating apoptosis, and it is reported that inhibition of CTSB during *in vitro* maturation (IVM) as well as *in vitro* culture (IVC) *via* E-64, one kind of CTSB inhibitor, could increase bovine and porcine blastocyst production (Balboula et al., 2010a; Bettegowda et al., 2008; Kim et al., 2015; Min et al., 2014). In addition, E-64 could reduce CTSB expression in embryo cells during IVC (Min et al., 2014). However, it remains unclear whether E-64 is effective to increase sheep blastocyst rate and the role of E-64 on CTSB expression in sheep cumulus cells.

In this study, we investigated the changes of CTSB transcript abundance, CTSB activity in sheep cumulus cells, apoptosis levels of cumulus cells during IVM, and the correlations between these indicies and the developmental competence of oocytes. Furthermore, we investigated changes of CTSB expression in sheep cumulus cells while

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CTSB activity was inhibited by E-64.

2. Materials and methods

All experimental protocols and animal handling procedures were reviewed and approved by the Laboratory Animal Care and Use Committee of Hebei Province. Small Tail Han Sheep were used for our experiments.

2.1. Experimental design

Five experiments were conducted in our study. In each experiment, cumulus oocyte complexes (COCs) in control group were cultured in E-64-free medium and COCs in experimental groups were cultured in medium containing 1, 5, or 10 μM E-64. In e*xperiment 1*, CTSB activity in sheep COCs at IVM intervals 0, 8, 16, or 24 h of IVM and the effects of 1, 5, or 10 μM E-64 on CTSB activity were evaluated. Experiment 1 was repeated six times with 50 COCs per replication. In e*xperiment 2*, CTSB activity in cumulus cells and oocytes at IVM 0 or 24 h was detected to confirm the CTSB activity in oocytes and cumulus cells. Experiment 2 was repeated eight times and 15–20 COCs were used per replication. *In experiment 3*, the relative expression of CTSB in sheep cumulus cells was detected. Experiment 3 was repeated six times with cumulus cells from 40 COCs per replication. *In* e*xperiment 4*, apoptosis rates in sheep cumulus cells were evaluated. Experiment 4 was repeated six times and cumulus cells from 100 COCs were used per replication. In e*xperiment 5*, we evaluated the effect of different concentrations (1, 5, and 10 μM) of E-64 on sheep *in vitro* fertilization (IVF) and parthenogenetic embryos *via* evaluation of cleavage rates and blastocyst rates. Experiment 5 was repeated six times and the numbers of oocytes used were shown in Tables 2 and 3.

2.2. Collection of COCs and IVM

Sheep ovaries were collected at a local abattoir from adult sheep (2–4 years old). Ovaries were washed in saline solution. The COCs were aspirated from 2 to 6-mm follicles and washed three times in TCM199 (Gibco, Grand Island, NY, USA) containing 2% (v/v) fetal bovine serum (FBS, Gibco). Twenty COCs were matured in 100-μL drops of maturation medium (TCM199) containing 10% (v/v) FBS, 10 μg/mL follicle-stimulating hormone (FSH, Bioniche, Belleville, ON, Canada), 10 μg/mL luteinizing hormone (Bioniche, Belleville, ON, Canada), 1 μg/mL oestradiol (Sigma-Aldrich), 1 mM L-glutamine (Sigma-Aldrich), 20 ng/mL epidermal growth factor (EGF, Sigma-Aldrich), 100 μM cysteine (Sigma-Aldrich), and 40 mM sodium pyruvate (Sigma-Aldrich). The CTSB inhibitor, E-64 (N-[N-(L-3-*trans*-carboxirane-2-carbonyl)-L-leucyl]-agmatine, Sigma-Aldrich), was added to the maturation medium at different concentrations (1 μM, 5 μM or 10 μM). The E-64 was dissolved in embryo-tested water, and aliquots were stored at −20 °C. The culture media containing 1, 5 μM and 10 μM E-64 were prepared by adding 10 μL each of 100 μM, 500 μM and 1 mM E-64 stock solutions per milliliter of maturation medium, respectively. Similarly, the control medium (without E-64) was prepared by adding 10 μL of embryo-tested water per milliliter of maturation medium. The drops were covered by mineral oil (Sigma-Aldrich) and incubated for 24 h in air containing 5% CO_2 with maximum humidity at 38.5 °C.

2.3. IVF

Frozen semen (from one fertility-proven ram) was thawed by immersing in water (38 °C) for 30 s, and then was placed in a tube. The tube was incubated in air containing 5% CO_2 with maximum humidity at 38.5 °C for 40 min after addition of 2 mL of HEPES SOF (synthetic oviductal fluid). Subsequently, 1 mL of the supernatant from this tube was pipetted and centrifuged (2 min at 2000*g*)at 25 °C. After removing the supernatant, sperm was appropriately diluted with SOF containing 20% (v/v) FBS and 10 μg/mL heparin sodium (Sigma-Aldrich) to obtain a concentration of 1×10^6 to 3×10^6 sperm/mL. Twenty microliters of the sperm suspension was added to 15 COCs with one drop (30 μL) of SOF containing 20% (v/v) FBS under mineral oil. The COCs and sperm were coincubated for 24 h. Thereafter, gametes were washed three times with embryo culture medium consisting of SOF supplemented with 1% (v/v) eagle basal medium (Sigma-Aldrich), 1%(v/v) minimum essential medium (Sigma-Aldrich), 8 g/L BSA (bull serum albumin, Sigma-Aldrich), 10 ng/mL EGF and 1 mM L-glutamine.

2.4. Parthenogenetic activation of oocytes

After IVM for 24 h, cumulus cells were removed from oocytes through gentle pipetting in Dulbecco's phosphate-buffered saline (DPBS, Gibco) containing 0.1% hyaluronidase (Sigma-Aldrich). The oocytes then were washed three times in DPBS. Oocytes were activated in DPBS containing 2.5 μM ionomycin (Sigma-Aldrich) and 3% (w/v) BSA for 3 min. The oocytes then were incubated in embryo culture medium supplemented with 2 mM 6-dimethylaminopurine (Sigma-Aldrich) for 4 h in air containing 5% CO_2 with maximum humidity at 38.5 °C.

2.5. In vitro culture and fluorescence staining of embryos

A total of 15–20 fertilized embryos or parthenogenetic embryos were placed in 50-μL drops of embryo culture medium covered by mineral oil and cultured in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 38.5 °C. The day of IVF was considered Day 0 and the day of parthenogenetic activation was considered Day 1. Cleavage rates, blastocyst rates, and blastocyst hatching rates were each evaluated on Day 2 and Day 8.

Blastocysts were washed three times in DPBS and stained with 10 μg/mL Hoechst 33342 (Sigma-Aldrich) at 38.5 °C for 10 min. Blastocysts were mounted on glass slides in a 10-μL drop of glycerol and squashed gently with cover slips following a final washing. Finally, blastocysts were observed under a fluorescent microscope (IX71, Olympus, Japan) and the numbers of stained cell nuclei were counted.

2.6. Detection of CTSB activity in oocytes and cumulus cells at 0 h and 24 h of IVM

CTSB activity in cumulus cells and oocytes was detected using the Magic Red Cathepsin B Detection Kit $(MR\text{-}RR)_2$ (LLC, P 6133; Immunochemistry Technologies, Bloomington, MN, USA) according to the manufacturer instructions. In brief, COCs were stained with 2 μL reaction mix in 500 μL dimethyl sulfoxide (Sigma-Aldrich) and were incubated for an additional 30 min at 37 °C under 5% CO_2 in a humidified atmosphere. Five microliters of Hoechst 33342 were added at a concentration of 10 μg/mL and COCs were incubated under identical conditions for 10 min. Images were taken under a laser-scanning confocal microscope with Alexa Fluor 594 and Alexa Fluor 405 separately and were analysed with image analysis software (Image pro-Plus, Media Cybernetic, Rockville, MD, USA).

2.7. Detection of CTSB activity in COCs

COCs and 500 μL of DPBS were added into centrifuge tubes and the supernatant was removed after centrifugation. CTSB activity was detected using the Cathepsin B Activity Assay Kit (Abcam, Cambridge, UK). In brief, samples were incubated on ice for 15 min after the addition of 25 μL of cell lysis buffer. The protein concentration of each sample was determined with the bicinchoninic acid method. The supernatant containing 50 μg of protein was transferred to each well of a 96-well plate, and different volumes of cell lysis buffer were added to reach a total volume of 50 μL. Subsequently, 50 μL of reaction buffer and 2 μL of substrate Ac-RR-AFC (10 mM) were added to each