The complex roles of autophagy and lysosome-cathepsin on the oocyte maturation and preimplantation embryo development in cattle

（ウシ卵子成熟および初期胚発生に及ぼすオートファジーおよびリソソームカテプシンの複合的影響）

北海道大学　大学院農学院
生物資源科学専攻　博士後期課程

Jianye LI

Contents

Abbreviations

ART	Assisted reproductive technologies
ATG	Autophagy related gene
Bp	Base pair
cDNA	Complementary DNA
COC	Cumulus cell complex
CTSs	Cathepsins
CTSB	Cathepsin B
DMSO	Dimethyl sulfoxide
ET	Embryo transfer
H2AFZ	Histone H2A family member Z
ICM	Inner cell mass
IVC	*In vitro* culture
IVF	*In vitro* fertilization
IVP	*In vitro* produced
IVM	*In vitro* maturation
LC3	Microtubule-associated protein light chain 3
mRNA	Messenger RNA
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PVA	Polyvinyl alcohol
Rapa	Rapamycin
RT-qPCR	Real time quantitative polymerase chain reaction
TUNEL	Terminal deoxynucleotidyl transferase biotin-dUTP nick end Labeling

TE	Trophectoderm

Unit measures abbreviations

%	Percentage
Min	Minute
Ml	Milliliter
Ng	Nanogram
Sec	Second
H	Hour
× *g*	Times gravity
°C	Degree Celsius
μM	Micromolar
Mg	Microgram
ml	Microliter
Mm	Micrometer
W/V	Weight by volume
V/V	Volume by volume
IU	International unit
Mm	Millimeter

Abstract

Assisted reproductive technologies (ART), such as *in vitro* fertilization (IVF), offer great potential for improving the productivity of domestic animals, including cattle. However, the overall reproductive efficiency of *in vitro* produced (IVP) embryos remains lower than *in vivo* produced embryos. Therefore, a comprehensive understanding of the molecular and cellular functions is necessary to assess embryo quality prior to embryo transfer (ET) and to improve the pregnancy rate. Many approaches have been achieved to evaluate the oocyte quality and competence to support embryo development. Recent studies have revealed the important roles of lysosomal cathepsin B (CTSB) and autophagy in development of preimplantation embryos. However, little is known about the effect of lysosome-cathepsin and autophagy on the quality and developmental competence of oocytes.

In this study, I investigated the roles of lysosome-cathepsin and autophagy on the oocyte maturation and preimplantation embryo development in cattle.

Bovine cumulus oocyte complexes (COCs) collected from ovaries were classified into good-quality and poor-quality based on morphological criteria. Then good and poor COCs were subjected to *in vitro* maturation (IVM), IVF followed by *in vitro* culture (IVC) for 8 days. The blastocyst quality was confirmed by total cell number and TUNEL-positive cells. The total cell number of blastocysts derived from poor quality

COCs was lower than those from good quality COCs. In addition, higher TENEL positive cells were detected in blastocysts derived from poor quality COCs compared to those in good quality. Autophagic activity of matured oocytes was detected with fluorescent dye both in poor and good quality oocytes. Autophagic activity was significantly lower in poor quality oocytes than good quality oocytes. Immunostaining of an autophagy protein marker, microtubule-associated protein light chain 3 (LC3), also revealed that in poor quality oocytes, lower number of LC3-stained particles were detected than those of in good quality ones. Autophagy inhibition decreased developmental competence together with increase in CTSB activity and *caspase 3* (an apoptosis-related gene) expression. These results suggest that the quality and developmental competence are negatively correlated with autophagy associated with CTSB in bovine oocytes. In previous studies, autophagy and CTSB have revealed crucial roles on developmental competence of COCs. Thus, I investigated the effect of autophagy induction and CTSB inhibition using an autophagy inducer; rapamycin, a CTSB inhibitor; E-64, and combined administration in poor quality COCs during IVM. Rapamycin significantly increased the autophagic activity of poor quality oocytes. CTSB inhibition by E-64 also increased autophagic activity together with expression of autophagy related genes (*LC3α*, *LC3β*). These results suggest that autophagy induction is regulated not only by rapamycin, but also by CTSB mediated pathway. Single administration of rapamycin significantly increased blastocyst rate after IVF and IVC of poor COCs with increasing total cell number and decreasing TUNEL-positive cells,

whereas positive effect was not clearly observed in good COCs. In contrast, combined administration of rapamycin and E-64 significantly increased blastocyst rate, total cell number, and decreased TUNEL-positive cells both in good and poor quality COCs. These results suggest that autophagy induction has a potential rescue effect on developmental competence of poor quality COCs, and combinational effect of CTSB inhibition and autophagy induction during IVM also have promoting effect on developmental competence both in poor and good quality COCs. Recent trend on the increased demands of IVP embryos used for ET has revealed the lack of supply of oocytes and ovaries. Thus, oocyte retrieve from ovaries of cows from local abattoir in a distant place became important. However, decreased quality of COCs for a long time preserved ovaries causes low developmental competence. Therefore, improving the developmental competence of preserved COCs is an urgent issue. The present research was conducted to investigate the effect of autophagy and CTSB regulation on developmental competence of bovine oocytes collected from transported ovaries. COCs collected from preserved and transported ovaries at 20°C for 24 h were used for IVM with or without rapamycin and/or E-64, followed by IVF and IVC. Developmental competence of COCs from transported ovaries showed a low blastocyst rate after IVM, IVF, and IVC compared with freshly collected COCs. Single administration of E-64 or rapamycin did not affect blastocyst rate, however, mixed supplementation of E-64 and rapamycin significantly increased the blastocyst rate with increased total cell number. These results indicate that the synergetic effect of regulation of autophagy induction and

CTSB inhibition can improve embryo development derived from long-transferred ovaries.

In conclusion, autophagy can be used for one of the potential markers for evaluation of oocyte quality. In addition, the synergetic effect of CTSB inhibition and autophagy induction can contribute to improve quality and developmental competence of oocytes for ET in cattle.

Chapter 1 **General introduction**

Embryo transfer (ET) refers to a step in the process of assisted reproduction in which several embryos are placed into the female uterus with the intent to establish a pregnancy. This technique has great potential as a reproductive technology for domestic animals and becomes one of the prominent high businesses worldwide. ET has become one of the most powerful tools for animal reproductive scientists and breeders to improve their animal herds and increase the number of superior animals in a short time.

Assisted reproductive technology (ART), such as *in vitro* maturation (IVM), *in vitro* fertilization (IVF), offers great potential for improving the reproductive efficiency of domestic animals. However, the overall efficiency and viability of *in vitro* produced (IVP) embryos remain lower than those of *in vivo* embryos [1,2]. Not all of the putative zygotes obtained from IVM and IVF have the ability to develop to blastocyst stage. In ART, the developmental competence is determined by the quality of the oocytes and blastocysts produced following a long period of IVM and following development [3]. Even though many researchers have been making lots of efforts to promote embryo quality and viability, there are still many limitations of developmental competence and viability of the IVP embryos. Thus, it is important to find and prevent the risk factors of oocyte maturation and embryo development to improve the culture system as well as the developmental competence and quality prior to ET.

In general, oocyte and embryo quality are evaluated by their morphology [4].

However, morphological evaluation does not correlate with the quality of embryos perfectly. Thus, it is important to understand the regulatory mechanisms of the quality of oocyte and preimplantation embryos.

Macroautophagy (simply referred to as autophagy hereafter) is a multiple process by which the cytoplasmic components degraded by lysosomal proteases. In autophagy processes, cytoplasmic constituents, including organelles like mitochondria, are firstly enwrapped by a membrane sac which named isolation membrane. Next, the closure of the isolation membrane results in the formation of autophagosome, which has double membrane structures, known as the initial autophagic vacuoles. The following step is the fusion of autophagosomes and endosomes, resulting in the formation of amphisomes or intermediate autophagic vacuoles. Finally, after fusion of the outer membranes of these autophagosomes and lysosomes, the autolysosomes or degrading autophagic vacuoles are generated [5–8]. Lysosomal hydrolases degrade the cytoplasm derived components of the autophagosome, together with its inner membrane. Once macromolecules are degraded in the lysosomes, the monomeric units (like amino acids) are exported to the cytosol for reuse [9]. In simple terms, autophagy includes several sequential steps: sequestration, transport to lysosomes, degradation and utilization of degradation products. These step-dependent functions may allow autophagy have multiple functions. Recent researches have clearly demonstrated that autophagy has a wide variety of physiological and pathophysiological roles, such as starvation adaptation, intracellular protein and organelle clearance, anti-aging, elimination of

microorganisms, cell death, tumor suppression, antigen presentation and development [10].

Systemic and tissue-specific autophagy-related gene (*Atg*) knockout mice provided great evidences of pivotal roles on autophagy in mammalian development and cell differentiation [11]. For example, in embryogenesis, knockout mice of *Atg* gene (*Atg 3, 5, 7, 9* and *16*) causes neonatal lethality despite almost normal appearance at birth [11]. Deletion of *Atg5* leads to lethality during mid-embryonic development. In addition, the *Atg5, 7* knockout mice were born at Mendelian ratio, and the major organs were almost normal, however they display some developmental abnormalities, and died within 1 day [12,13]. The autophagy-mediated machinery is essential for two critical period of early development: the preimplantation period after oocyte fertilization in mice [14], and the early postnatal period after disruption of the placental nutrient supply. In addition, autophagy also plays an important role in other stages of embryogenesis. The basic role of autophagy is to produce necessary amino acid and to maintain the intracellular homeostasis by elimination misfolded proteins and abnormal organelles delivered to lysosomes, resulting in degradation of cytoplasmic content by lysosomal proteases [15].

Lysosome contains considerable number of proteases. Among them the well-known are lysosomal cathepsins (CTSs) family of the proteases. Many kinds of CTSs have been identified in animals and categorized into the three distinct groups according to their amino acid residue in the active site, which included: Serine cathepsins (including CTSA and CTSG); Cysteine cathepsins (including CTSB, C, F, K, L, O, S, V, X and W);

Aspartic cathepsins (including CTSD and CTSE). The cysteine CTSs are predominantly endopeptidases, which are located intracellularly in endo-lysosomal vesicles [16]. Lysosomal CTSs are the member of papain family and have been found in all of the organisms in mammals. CTSs play an essential role in the proteolytic mechanism; the majority of CTSs function is to participate in degradation and recycling of proteins within the lysosome. Not only that many CTSs have been also found to play the important roles in distinct physiological processes, induce intracellular protein turnover, immune response, bone remodeling, proprotein processing, antigen-presentation, degradation of extracellular matrix as well as initiation of cell death. Importantly, cysteine CTSs play their hydrolytic intracellular digestion at acidic pH, because they are optimally active in a slightly acidic pH and are mostly unstable at neutral pH. Since CTSs are outside the lysosomes or extracellularly they can be relatively rapidly irreversibly inactivated at neutral pH [17].

Among CTSs family, CTSB is known to play an important role in intracellular protein catabolism, it entered the lysosomal system from outside of the cell via endocytosis, phagocytosis, or autophagy [18]. CTSB is a glycoprotein with molecular weight of 30 kDa containing disulfide-linked heavy and light chain submit [19]. CTSB is initially synthesized as the inactive zymogen, and post-translationally processed into its active configurations after subsequent incorporation into the acidic environment of the lysosomes [20]. Moreover, the expression of CTSB is detected in many type of cells and tissues, including liver, ovary, cumulus cells, and oocytes [21–23].

Programmed cell death (PCD), or apoptosis plays a central role in the development and homeostasis of metazoans [24–28]. Dysregulation of apoptosis results in a variety of pathologies including cancer, autoimmune diseases, and neurodegenerative disorders [29–31]. Recently, the accumulating evidences suggest that PCD is not confined to apoptosis but the cells through different pathways for active self-destruction as reflected by the morphology; apoptosis, autophagic cell death, etc. Autophagic cell death activated when massive removal of cells or cytoplasm is demanded like developmental programmes [32]. In addition, the accumulating evidences suggest that lysosomes are important mediators of PCD. Proteases released from the lysosomal compartment trigger initiating events of apoptosis. Furthermore, lysosomotropic agents may be controlled by lysosomal cysteine proteases (cathepsins) to caspases therefore active apoptosis [32].

Caspases are the central components of the apoptotic response [33,34]. Caspases are lysosomal cysteine proteases that cleave after aspartate residue in their substrates [35] and conserved family of enzymes that irreversibly leads to cell die. Up to date, there are at least 14 distinct caspases have been identified in mammals, of which 11 caspases are included in human. In general, apoptotic caspases are divided into two classes in mammals: 1) the initiator caspases, which include caspase 2, 8, 9 and -10; 2) the effector caspases, which include caspases 3, 6 and -7 [36]. The schematic diagrams of lysosomal cell death pathways are shown in Fig. 1, according to previous review with slight modification [37].

In my master thesis, I have found that the expression levels of *CTSB* and *Beclin 1* (an autophagy related gene) transcript were significantly higher in metaphase II (MII) oocyte stage followed by a significant decrease after 8-cell embryo stage. Moreover, in the blastocyst stage, *CTSB* transcript showed significantly higher in trophectoderm (TE) than inner cell mass (ICM). Consistent with the qRT-PCR profile of *CTSB*, CTSB activity supported by the lysosomal activity showed the highest activity just after fertilization (1-cell embryo) and at the morula stage among the other developmental stages. However, immunolocalization of CTSB did not show the similar pattern of CTSB and lysosomal activities, where CTSB protein was clearly localized and significantly increased in morula and blastocyst stages. In contrast to qRT-PCR results, CTSB and lysosomal activity and protein expression were significantly higher in ICM than TE both in intact and separated blastocysts.

In addition, I also found that autophagic activity followed the same pattern of CTSB and exhibited the highest activation immediately after fertilization. Again, similar to CTSB and lysosomal activity, autophagic activity was significantly higher in ICM when compared to that of TE. These findings suggested that maternal CTSB and autophagy might have essential roles at the start point of both embryonic development and differentiation. Therefore, my master course study suggested that autophagy mediated lysosomal CTSB might have a pivotal role during embryonic development and differentiation.

Recent studies have revealed the important roles of lysosomal CTSB and autophagy

in development of preimplantation embryos [14,38]. However, little is known about the effect of lysosome-cathepsin and autophagy on the quality and developmental competence of oocytes.

In this study, I investigated the effect of lysosome-cathepsin and autophagy during IVM on preimplantation embryo development in cattle.

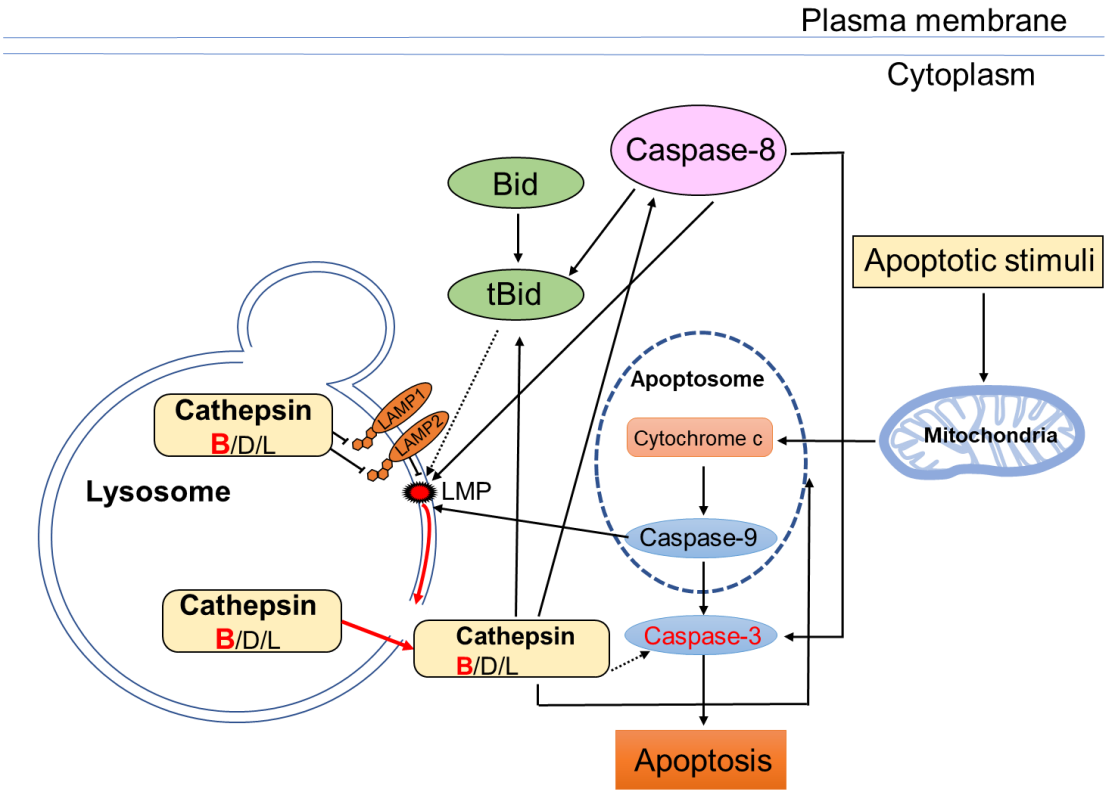

Fig. 1. Schematic diagrams of lysosomal cell death based on Aits S *et al.* (J Cell Sci, 2013) with slight modification.

Abbreviations: Bid: BIH3-interacting domain death agonist; LAMP: lysosome-associated membrane protein; LMP: lysosomal membrane permeabilization; tBid: truncated Bid.

Chapter 2 **Relationship between the quality and autophagic activity in bovine oocytes**

Introduction

The process of oocytes after fertilization, to develop to the blastocyst stage, to attach to the maternal uterus, and to generate a healthy offspring is not a simple. Many strategies have been employed to improve the efficiency of IVP of cattle embryos [39]. However, the overall embryo production efficiency remains lower than that of *in vivo* ones. Although many factors affect the efficiency and quality of IVP technology, the most important factors are the physiological condition of the donor and the IVC conditions. General factors such as age, donor conditions, and herd management play an important role together with some specific factors like reproductive health and ovarian cyclicity. Among them, the oocyte quality is a major factor. Oocyte quality highly determines early embryonic survival, developmental competence, establishment and maintenance of pregnancy, fetal development and adult health [40]. Therefore, it is necessary to modulate the IVM system to rescue poor quality oocytes with subsequent improvement in the efficiency and quality of IVP embryos.

At the time of oocyte collection for IVM, the quality of COCs is generally evaluated by their morphological criteria [4]. The morphological features such as thickness of the

cumulus layer and status of oocyte cytoplasm are still the most commonly used criteria for classification of the immature COCs quality [41]. Briefly, the COCs with compact and complete cumulus layer surrounding the oocytes, and the cytoplasm of oocyte slightly homogenous and those medium brown in color were considered as good quality. In contrast, the COCs showing the abnormal cumulus expansion and ooplasmic features were classified as poor quality.

The bi-directional communication between the oocyte and cumulus cell occurs throughout follicular development [42] and is essential in the acquisition of developmental competence of mammalian oocytes. The physiological importance of the role of gap junctions between oocytes and cumulus is well known [43]. Cumulus cells play important roles, particularly in normal cytoplasmic maturation oocytes, regulation of oocyte metabolism, and protection of oocytes from harmful environments [44–47]. Moreover, cumulus cells stimulate glutathione synthesis in bovine [48–50] and prevent harmful effect induced oxidative stress during IVM [51]. For these reasons, morphologically poor oocytes that have less cumulus cells than morphologically good oocytes are discarded. However, some rate of poor quality oocytes still have potential competence that develop to blastocyst and use for ET. Therefore, it is important to clarify the factors to evaluate oocyte quality and improve the developmental competence by controlling the factors to produce IVC embryos and subsequent ET. Among the several factors affecting oocyte quality and developmental competence, autophagy has been recently focused on one of factors [15]. Autophagy is an

evolutionarily conserved catabolic process, in which misfolded proteins or abnormal organelles engulfed by autophagosomes and delivered to lysosomes, resulting in the degradation of cytoplasmic component by lysosomal proteases [52].

The basic roles of autophagy are not only the simple elimination of materials, but a dynamic recycling system that production of necessary amino acids, produces new energy for cellular renovation and maintenance of normal intracellular homeostasis. In addition, autophagy is involved in many other processes, including cancer, aging, cell death, viral infection, and development [53]. Autophagy plays a critical role during fertilization and is essential for normal embryonic development and differentiation in mice [14,15]. In fact, it has been demonstrated that autophagy is highly induced at the time of fertilization, and autophagy-deficient embryos are arrested before blastocyst stage [14]. The embryos derived from autophagy-related gene 5 (*Atg5*) knock-out oocyte fertilized with *Atg5*-null sperm develop arrested at the 4- and 8-cell embryo stage but could develop if they were fertilized by wild-type sperm [14]. In autophagy mediated molecular signal pathway, microtubule-associated protein light chain 3 (LC3) is one of the autophagy protein markers. LC3 present at both inner and outer sides of autophagosomes. In LC3 protein family, the cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine to form LC-phosphatidylethanolamine conjugate (LC3-II), which is recruited for autophagosomal membranes [54]. Importantly, autophagy has been demonstrated as an indicator for selecting good quality embryos of mice by imaging the fluorescence intensity of green fluorescent protein fused to the

N-terminal LC3 (GFP-LC3, an autophagy marker) showed that autophagic activity was declined in poor quality embryos, probably because of a decline in the activity of lysosomal hydrolases [15].

Lysosomal CTSs are ubiquitous proteases, which belong to the aspartic, cysteine or serine protease families, and catalyze the hydrolysis of proteins. The cysteine CTSs are predominantly endopeptidases, which are located intracellularly in endolysosomal vesicles. CTSs regulate variety of normal biological processes such as cell death, proliferation, migration, protein turnover and cancer [55]. In particular CTSB plays in important roles in implantation, pregnancy and embryonic development [56]. Importantly, CTSB emerged as a key player in the initiation of the apoptotic pathway due to activation of initiator caspases rather than executioner caspases directly or indirectly in mouse fibroblast L929 cells [56]. Apoptosis includes a lot of membrane receptors and a signal transduction cascade, leading to the activation of several cysteine proteases known as caspases [57,58]. In the process of mammalian embryo development, apoptosis first appears in 32- to 64- cell embryos and is subsequently observed throughout embryogenesis [59]. Apoptosis occurs in blastocyst and is in reality crucial for further development, however, alterations to apoptosis in blastocyst can compromise development and may result in early embryonic death or fetal malformations [60]. Particularly, CTSB can activate caspases indirectly through inducing of mitochondrial membrane degradation, resulting in translocation of apoptosis-inducing components from the mitochondria to the cytosol [61]. In

consistence, recent study has revealed that inhibition of CTSB activity decreases apoptosis by preventing cytochrome c release from mitochondria in porcine parthenotes [62]. Giving the inverse relationship between apoptosis and embryo quality, it is plausible that higher CTSB activity was observed in poor quality and heat-shocked bovine oocytes when compared to control non heat-shock oocytes [41,63]. Inhibiting the CTSB activity in oocytes and embryos increased the developmental rate and the quality of embryos [64], indicating that lysosomal CTSB regulation is a promising strategy to improve the quality of IVP embryos.

However, role of autophagy on bovine COCs and oocyte quality and their subsequent development is still unknown in bovine oocytes. Therefore, I employed the following experiments: 1) evaluation of autophagy in COCs and oocyte quality. 2) inhibitory effect of autophagy on autophagic activity of COCs and oocytes and subsequent embryo development during IVM, and 3) effect of autophagy inhibition on CTSB activity, gene expression levels of *caspase 3* (apoptosis-related gene), *CTSB* of COCs and oocytes during IVM.

Materials and methods

Oocyte collection and *in vitro* Maturation (IVM)

Bovine ovaries were collected from a local abattoir and transported to the laboratory within 2 h. The ovaries were washed several times in 0.85% of saline followed by cutting the surrounding tissues. COCs were aspirated from follicles (2-8 mm in diameter) using a disposable 18-gague needle (Terumo, Tokyo, Japan) attached to a 10-ml syringe (Terumo). Aspirated follicular fluid was diluted in PB1 (supplementary list) medium and selected COCs were washed three times in the tissue culture medium-199 [TCM-199 (Gibco, Grand Island, NY)] (Supplementary list)]. The selected COCs were classified as two groups according to their morphological quality as described previously [41,65]. The quality of COCs evaluated based on the cumulus layer and the status of their cytoplasm. In brief, COCs with compact and complete cumulus layer and evenly granulated and homogenous cytoplasm were considered as good quality oocytes. By contrast, COCs showing abnormal cumulus distribution and ooplasmic features were classified as poor quality oocytes. The representative of selected morphological quality of COCs is shown in Fig. 2A. Selected COCs were matured in a 100 µl drop of TCM-199 (up to 20/ drop) using the 60mm dish (Becton Dickinson Labware, Franklin Lakes, NJ, USA), covered with liquid paraffin (Nacalai Tesque, INC, Kyoto, Japan) for 22 h in a humidified atmosphere containing 5% CO_2 in air. Then, collected COCs were removed from their surrounding cumulus cells in the presence of 0.1% hyaluronidase by gently pipetting to obtain the denuded germinal vesicle (GV) stage oocytes.

In vitro fertilization (IVF)

Frozen semen was thawed by immersing the straw in warm water (38.5˚C) for 30 sec. Subsequently, frozen-thawed semen was centrifuged at 600 *g* for 7 min in Brackett and Oliphant (B.O.) + theophylline working solution (Supplemental list) and 7.5 μg/ml heparin sodium salt (Nacalai Tesque). After removing the supernatant, the sperm pellet was diluted with IVF100 solution (Research Institute for the Functional Peptides, Yamagata, Japan) to prepare the final concentration of 5×10^6/ml of spermatozoa pellet to make 100 μl/drop. After washing trice in IVF100 medium, the matured good and poor quality COCs were transferred to IVF100 sperm medium (20 oocytes / drop) covered with liquid paraffin using the 60 mm dish and cultured for 18 h at 38.5 °C in a humidified atmosphere of 5% CO_2 and 5% O_2 in air, receptivity.

In Vitro Culture (IVC)

Followed by IVF, the cumulus cells attached on presumed zygotes were denuded mechanically by gently pipetting and cultured in 100 μl/drop of Bovine IVD101 (Research Institute for the Functional Peptides) containing 500 μl of 5% fetal bovine serum (FBS) (PAA Laboratories, QLD, Australia) and 5 μl of antibiotic stock, covered with liquid paraffin. Presumed zygotes derived from good and poor grade embryos were cultured in a humidified atmosphere of 5% O_2, 5% CO_2 in air and cultured for 8 days for subsequent analysis.

RNA extraction and quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA of twenty metaphase II (MII) oocytes or five COCs after IVM was isolated using a ReliaPrep RNA Cell Miniprep System (Promega, Madison, WI, USA) according to the manufacturer's instructions. The extracted RNA was immediately used for qRT-PCR or stored at −80˚C until analysis. After standardizing the RNA quantity using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), cDNA was then synthesized using the ReverTra, Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). On the other hand, cDNA from small number of oocytes (one or two) was synthesized with a Super Prep^{TM} Cell Lysis & RT Kit for qPCR (Toyobo, Osaka, Japan). Finally, qRT-PCR was performed to evaluate the expression of *CTSB* and *caspase3* transcripts relative to histone *H2AFZ* using Light Cycler® 480 (Roche, Basel, Switzerland). The primers used for the analysis are described in (Supplemental list, Table. 1). The reactions were carried out in 96-well PCR plates (Roche), in a total volume of 10 µl containing 5 µl of Thunderbird SYBR qPCR Mix (Toyobo), 1 µl of each primer (10 pmol/µl), and 3µl of cDNA, and then subjected to the following cycling conditions: one cycle at 95˚C for 30 sec (denaturation), followed by 55 cycles at 95˚C for 10 sec (amplification), 55˚C for 15 sec (primer annealing), and 72˚C for 30 sec (extension), a melting-curve step at a gradient of 55–95 ˚C with the increment of 2.2 ˚C/sec, and a cooling step at 4 ˚C. The relative expression levels of each of the target genes were calculated relative to that of internal control gene, histone family, membrane

Z (*H2AFZ*).

Detection of autophagic activity in good and poor quality COCs and (germinal vesicle) GV as well as spautin-1 treated MII oocytes and COCs

Autophagic activity in COCs and denuded GV stage oocytes was evaluated using Cyto-ID Green Autophagy Detection Reagent (Enzo Life Science, Farmingdale, NY), which is an prominent amphiphilic autophagosome tracer dye that measures the autophagic vacuoles and monitors autophagic flux in live cells, and co-localizes with LC3 and has negligible nonspecific staining of lysosomes [66,67] according to the manufacturer's protocol with minor modifications. In brief, after collection, COCs and denuded GV oocytes were incubated for 45 min in 500 μl 1x Assay Buffer (Enzo) including 1 μl/ml reaction mix at 38.5˚C in a humidified atmosphere of 5% CO_2 in airs. Nuclei were stained with 25 μg/ml Hoechst 33343 (Sigma-Aldrich, St. Louis, MO) added in the reaction solution. After incubation, the COCs and GV oocytes were washed three times in phosphate buffered saline (PBS) contain 0.2% polyvinyl alcohol (PVA) (0.2% PBS-PVA), followed by mounted onto a glass slide and observed under a fluorescence microscope using a 550-nm filter [(spautin-1 treated COCs and MII oocytes; as well as representative images of COCs by BZ-9000 Biorevo microscope (Keyence, Osaka, Japan), GV stage oocytes were captured by BZ-9000 Biorevo microscope or LAS X microscope (Leica, Germany), respectively]. The images were captured and fluorescence intensity was analyzed using the Image J software (National

Institutes of Health, Bethesda, MD, USA). Briefly, the defined cells were selected and the green cytoplasmic background was excluded by setting the threshold above the background level. This threshold was maintained at the same level for all analyzed images. The average total fluorescence intensity (pixels) was calculated to evaluate the autophagy activity and the number of particles (representing autophagosomes) was calculated.

Detection of intracellular activity of cathepsin B on COCs and oocytes after IVM

Detection of CTSB activity of COCs and oocytes were carried out using Magic Red CTSB detection kit (MR-RR)2 (immunochemistry Technologies LLC USA) according to the manufacturer's instructions at the indicated time points under constant fluorescence setting. In brief, a subset of COCs and oocytes were incubated in 300 μl of serum-free Dulbecco's modified Eagle medium (DMEM) with 1 μl of reaction mix at 37˚C in a humidified atmosphere of 5% CO_2 for 30 min, respectively. To stain nuclei, Hoechst was supplemented in a concentration of 25 μg/ml cultured in the reaction medium. After rinsing three times in 0.2% PVA-PBS, the freshly stained oocytes and embryos were mounted onto a glass-bottomed slide and immediately visualized under a BZ-9000 Biorevo fluorescence microscope (Keyence). Excitation filters of 365 nm and 590 nm were used for observing the nuclei and detecting intracellular CTSB activity, respectively. All images of CTSB activity were obtained using the same exposure time and the intracellular CTSB activity analyzed by Image J software (National Institutes of

Health). In brief, the defined cells were selected and the red cytoplasmic background was excluded by setting the threshold above the background level. This threshold was maintained at the same level for all analyzed images. The average total fluorescence emission (pixels) was calculated.

Immunohistochemical detection of LC3 protein in good and poor quality oocytes

After removing the zona pellucida by 0.1% (w/v) proteinase K (Sigma-Aldrich) in PB1 medium, the good and poor quality oocytes were washed three times in 0.2% PVA-PBS, respectively. Then, the oocytes were fixed by 4% paraformaldehyde (PFA) (Wako Pure Chemical Industries) dissolved in PBS for 1 h at room temperature. After washed three times for 5 min each in 0.2% PVA-PBS, the oocytes were permeabilized using PVA-PBS containing 0.2% Triton X 100 for 1 h at room temperature. Next, samples washed five times for 5 min each in washing solution containing 0.1% Triton X-100 and 0.3% bovine serum albumin (BSA) in PBS, the oocytes and embryos were incubated with a blocking solution of PBS containing 0.1% Triton X-100, 1% skim milk and 5% FBS for 1 h at room temperature and then incubated overnight at 4˚C with an primary antibody (Mouse Monoclonal antibody to LC3, M152-3Y, Medical and Biological Laboratories Co., LTD, Japan) diluted with 1:500 in blocking solution and control solution. After incubation with primary antibody, all samples were washed three times for 15 min each in washing solution, transferred to the fluorescein-conjugated secondary antibody (A1101, Anti-mouse IgG, polyclonal, Invitrogen, Tokyo, Japan)

diluted 1:400 in blocking solution and incubation for 1 h at room temperature. The validity of the immunostaining signal was confirmed by the negative control, which was incubated without the primary antibody. Finally, all samples were washed in washing solution for five times 5 min each followed by mounting onto glass slides using mounting solution (Vectashield with DAPI, Vector Laboratories, Burlingame, CA) and examined under the inverted confocal laser scanning microscope with a 40× immersion objective (LEICA TCS SP5, Germany). The obtained images were analyzed by Image J software.

Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assay

A TUNEL assay kit (*In Situ* Cell Death Detection Kit; Roche) was used to assess the presence of apoptotic cells in day 8 good and poor quality blastocysts derived from good and poor quality COCs, respectively. Blastocysts were fixed in 4% PFA for 1 h. After fixation, blastocysts were washed three times in 0.2% PVA-PBS, and then permeabilized in 0.2% PBS-PVA containing 0.5% Triton X-100 for 20 min. Blastocysts were washed three times in 0.2% PBS-PVA for 5 min each. The fragmented DNA ends were labeled with fluorescein-dUTP for 60 min at 37 °C in the dark. Hoechst 33342 (1 μg/ml, Sigma-Aldrich) was used to stain the nuclei. After incubation, the blastocysts were washed three times with 0.2% PBS-PVA for 5 min each, followed by mounting onto glass slides using mounting solution (Vectashield with DAPI, Vector Laboratories).

The fluorescence of the fragmented DNA was detected using a fluorescence microscope (LAS X, Leica) and a 550-nm excitation filter at 200×. The apoptotic index was calculated for each blastocyst as follows: apoptotic index = (number of TUNEL-positive nuclei/total number of nuclei) × 100.

Experimental design

After collection, COCs were divided into two groups as good and poor quality based on their morphology.

Experiment 1: Confirmation of embryo development and quality derived from good and poor quality COCs.

The good and poor quality COCs were subjected to IVM, IVF, and IVC until day 8. The developmental competence was evaluated by cleavage and blastocyst rate on day 2 and 8, respectively. Total cell number and TUNEL-positive cell ratio were used for assessing the embryo quality.

Experiment 2: Evaluation of autophagic activity LC3-stained particles in good and poor COCs and GV stage oocytes.

Good and poor quality COCs and denuded GV stage oocytes were subjected to investigation of autophagic activity by fluorescence dye (Cyto-ID) and LC-3-stained particles by immunostaining of LC3, respectively.

Experiment 3: Evaluation of inhibitory effect during IVM on autophagic activity in good quality COCs and oocytes and their subsequent development.

To investigate the inhibitory effect of autophagic activity on COCs and MII oocytes, a subset of the COCs were cultured with or without by an autophagy inhibitor, spautin-1 (10 μM) according to previous study [68] during IVM. After maturation, the COCs and denuded MII oocytes were used for evaluation of autophagic activity.

In addition, the remaining COCs divided into two groups during IVM: 1) TCM-199 alone (control group), 2) TCM-199 supplemented with 10 μM spautin-1. After IVF and culture for 8 days, cleavage and blastocyst rates were assessed on day 2 and 8, respectively. Total cell number were evaluated on day 8 blastocysts.

Experiment 4: Evaluation of relationship between autophagy inhibition and apoptosis, and CTSB activity during IVM.

A subset of COCs were cultured with spautin-1 during IVM to evaluate the effect of autophagy inhibition on relationship between autophagy inhibition, apoptosis, and CTSB activity. After IVM, CTSB activity and gene expression levels of *CTSB* and *caspase3* on COCs and denuded MII oocytes were employed.

Statistical analysis

Data are representative of at least three or five independent experiments. All data are shown as the mean ± standard error of the mean (SEM). Statistically significant differences were assessed by Student's *t-test* and one-way analysis of variance (ANOVA)-Tukey's Multiple Range Test implemented in Graphpad Prism® 7 Software (La Jolla, CA, USA). All percentage data were arcsine transformed prior to statistical

analysis. *P* values < 0.001, < 0.01, or < 0.05 were considered statistically significant. *P* values < 0.1 were regarded as indicating a tendency.

Results

Confirmation of oocyte quality by evaluation of blastocyst quality

The embryo cleavage rate and blastocyst rate were significantly higher derived from good quality COCs than those from poor quality COCs (Fig. 2B). The day 8 blastocysts derived from good COCs showed preferable morphology (Fig. 3A) and lower TUNEL-stained particles compared to those derived from poor quality COCs (Fig. 3B). In addition, the total cell number of blastocysts was significantly ($P < 0.05$) higher in blastocysts derived from good quality COCs than in poor quality ones (Fig. 4A), which was supported by the apoptotic index (Fig. 4B) that showed a high tendency ($P < 0.1$) in blastocysts derived from poor quality COCs compared to that in the good quality group.

Detection of autophagic activity in COCs and GV oocytes classified as good and poor quality

Autophagic activity was evaluated in good and poor quality COCs and GV oocytes using Cyto-ID dye. The fluorescence intensity of Cyto-ID reflects the autophagy flux. Autophagic activity was clearly observed in the cytoplasm of all COCs and oocytes. A stronger fluorescence of autophagic activity was observed in good COCs (Fig. 5A) than in poor quality COCs (Fig. 5B). In addition, the same pattern of fluorescence of autophagy in GV stage oocytes was observed in good quality oocytes (Fig. 6A and C and Fig. 7A) than poor quality oocytes (Fig. 6B and D and Fig. 7B). The intensity of autophagic fluorescence was significantly higher ($P < 0.001$) in good quality oocytes

than in poor quality oocytes (Fig. 8), which was corresponded with the number of particles representing autophagosomes that was significantly ($P < 0.05$) smaller in poor oocytes than good oocytes (Fig. 9).

Immunohistochemical detection of LC3 in good and poor quality oocytes

To evaluate whether the dynamic changes of autophagic activity in good and poor quality oocytes were accompanied by a difference in the autophagosomes localization of LC3, immunostaining of LC3 was performed in good and poor quality oocytes. Larger number of LC3-stained particles (representing autophagosomes) was detected in good quality oocytes than in poor quality oocytes (Fig. 10), and the number of LC3-stained particles was significantly ($P < 0.05$) larger in good quality oocytes compared to that in poor quality oocytes (Fig, 11).

Evaluation of inhibitory effect of autophagic activity on good quality COCs and oocytes

To investigate the inhibitory effect of autophagic activity on matured COCs and oocytes, the COCs were matured with or without by a specific and potent autophagy inhibitor, spautin-1 for 22 h. After maturation, the matured COCs and denuded oocytes were used for evaluation of autophagic activity. The autophagic activity was detected with Cyto-ID fluoregenetic substrate. The weaker green fluorescent signals were observed in the cytoplasm of both COCs and oocytes. The lower activity in COCs (Fig.

12B) and oocytes (Fig. 13B) after spautin-1 treatment was detected in the cytosol than non-treated COCs (Fig. 12A) and oocytes (Fig. 13A). The fluorescent intensity was significantly decreased in both COCs ($P < 0.05$) (Fig. 12E) and oocytes ($P < 0.001$) (Fig. 13E) compared to control groups.

Inhibitory effect of autophagy during IVM on developmental competence of oocytes

To investigate the inhibitory effect of autophagy during IVM on developmental competence of oocytes, 10 μM of spautin-1 was added into IVM medium. Autophagy inhibition during IVM affected the cleavage and blastocyst rate. Both cleavage ($P < 0.001$), blastocyst rates ($P < 0.05$) were significantly decreased after spautin-1 inhibition as well as total cell number ($P < 0.01$) of blastocysts (Table. 1).

Inhibitory effect of autophagy during IVM on CTSB activity of COCs

To understand relationship between autophagy inhibition and lysosomal CTSB activity, 10 μM of spautin-1 was supplemented into IVM medium for 22 h. CTSB fluorescence was stronger after spautin-1 treatment (Fig. 14B) of the COCs than control group (Fig. 14A). The fluorescence of CTSB (representing CTSB activity) was significantly ($P < 0.001$) higher in COCs with inhibitor compared to control COCs after IVM (Fig. 15).

Inhibitory effect of autophagy during IVM on *CTSB* and *caspase 3* gene expression of COCs and oocytes

To clarify the molecular interrelationship between autophagy inhibition and apoptosis mediators, 10 μM of spautin-1 was supplemented into IVM medium for 22 h. Spautin-1 treatment resulted in the mRNA levels of *CTSB* and *caspase 3* significantly increasing in COCs (Fig. 16) and oocytes (Fig. 17) compared to control group.

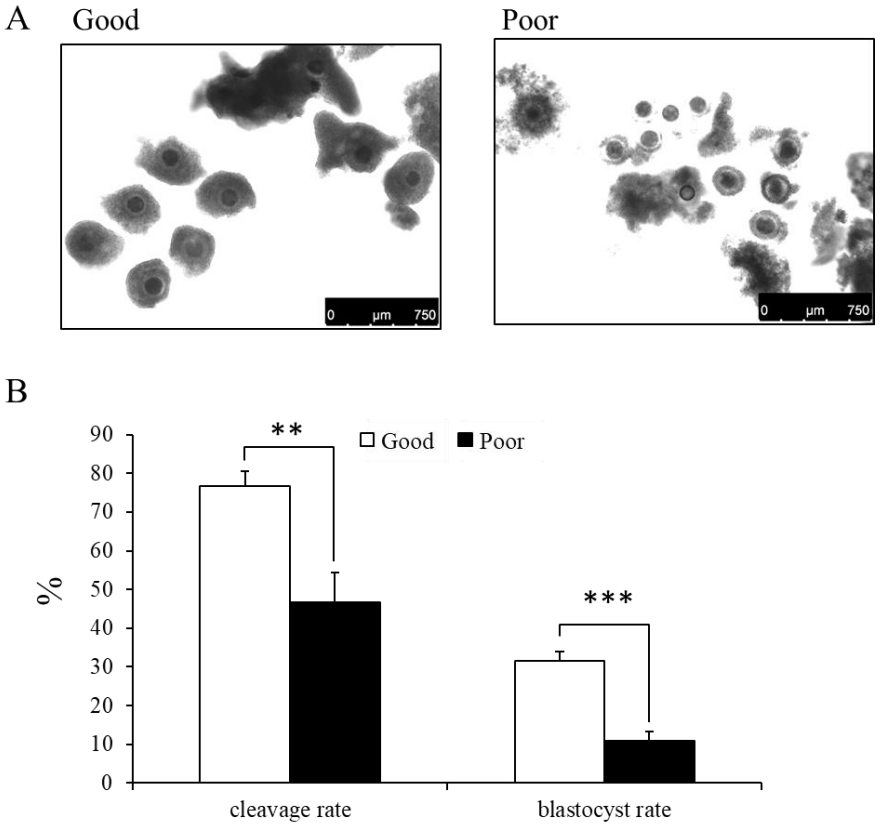

Fig. 2. Developmental competence of good and poor quality COCs. Representative photographs of good and poor quality COCs (A). COCs with compact and complete cumulus layer surrounding the oocytes, the cytoplasm is slightly homogenous and those medium brown in color were classified as good quality (left panel). COCs showing abnormal cumulus expansion and ooplasmic features were considered as poor quality (right panel). Cleavage rate and blastocyst rate (B) derived from good and poor quality COCs were evaluated on day 2 and day 8, respectively. The experiment was repeated five times. The scale bar represents 750 µm. All data are shown as mean ± SEM. Asterisks indicate statistical difference (**$P < 0.01$, ***$P < 0.01$).

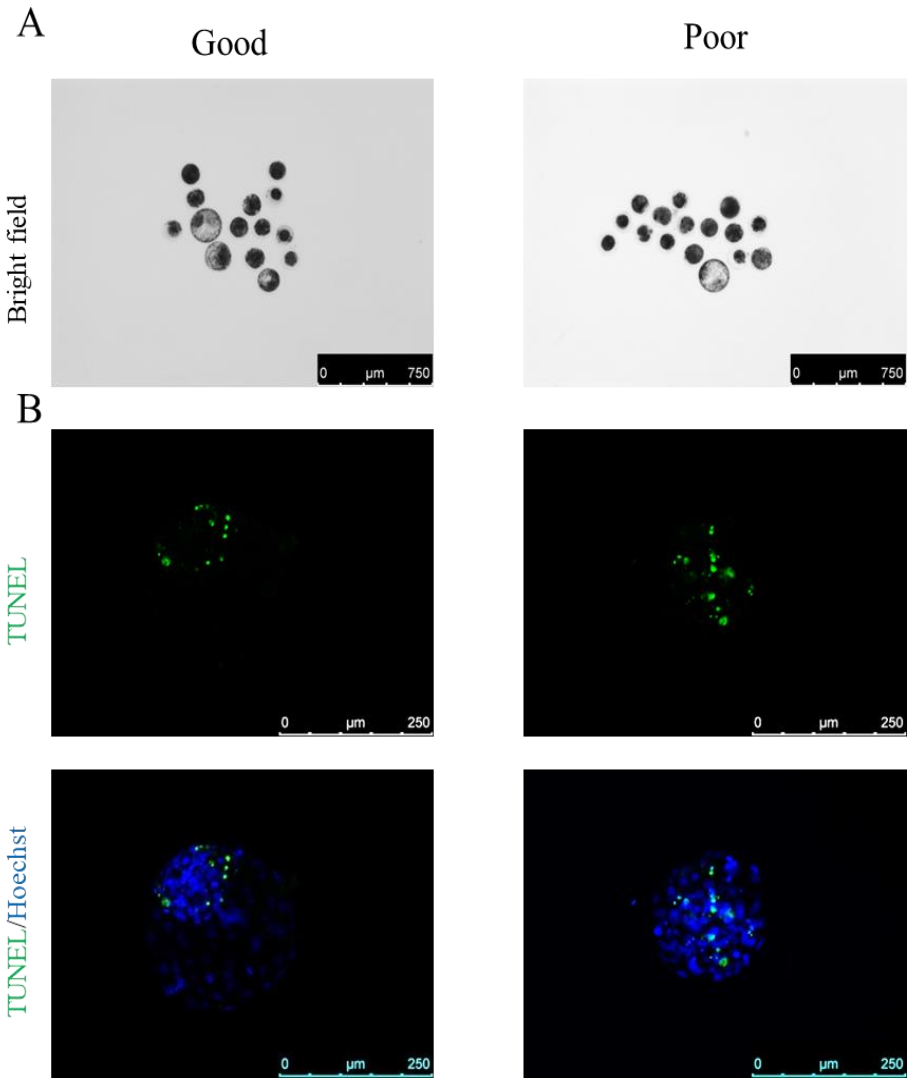

Fig. 3. Blastocysts derived from good and poor quality COCs. Representative photographs of blastocysts (A) derived from good and poor quality COCs and TUNEL staining of blastocyst (B). Light green dots show the TUNEL and blue color show the Hoechst 33342 for nuclei. The merged images are presented in green (TUNEL) and blue (Hoechst 33342). The scale bars represent (A): 750 µm and (B): 250 µm, respectively.

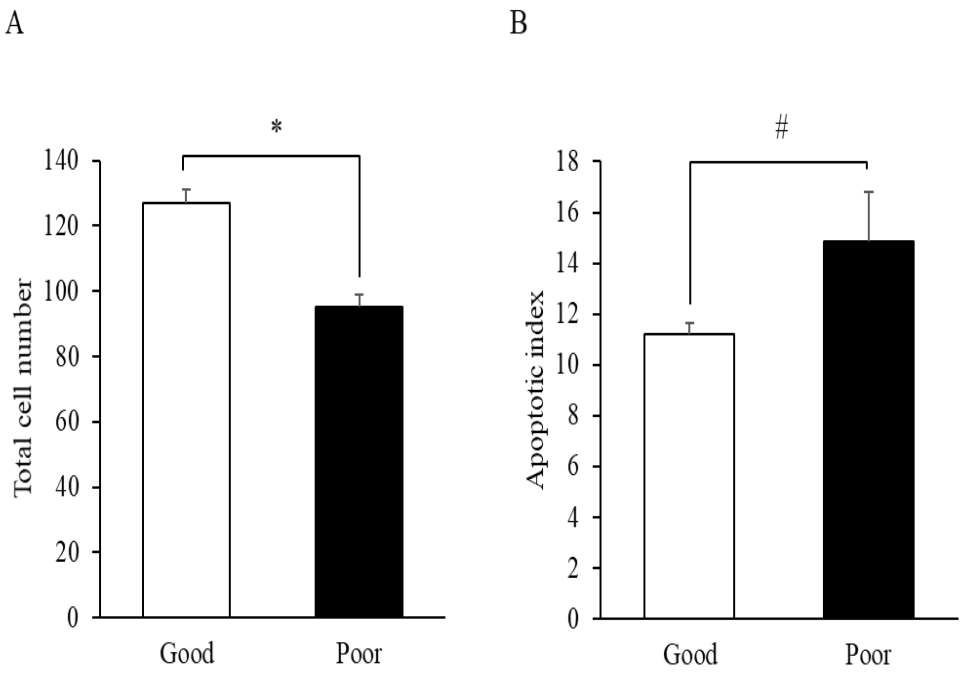

Fig. 4. Confirmation of validation of embryo quality derived from good and poor quality COCs by comparing the total cell number (A) and apoptosis levels (B). The experiment was repeated five times. All data are shown as mean ± SEM. An asterisks and number sign indicate statistical difference (*$P < 0.05$, #$P < 0.1$) between good and poor quality blastocysts, respectively.

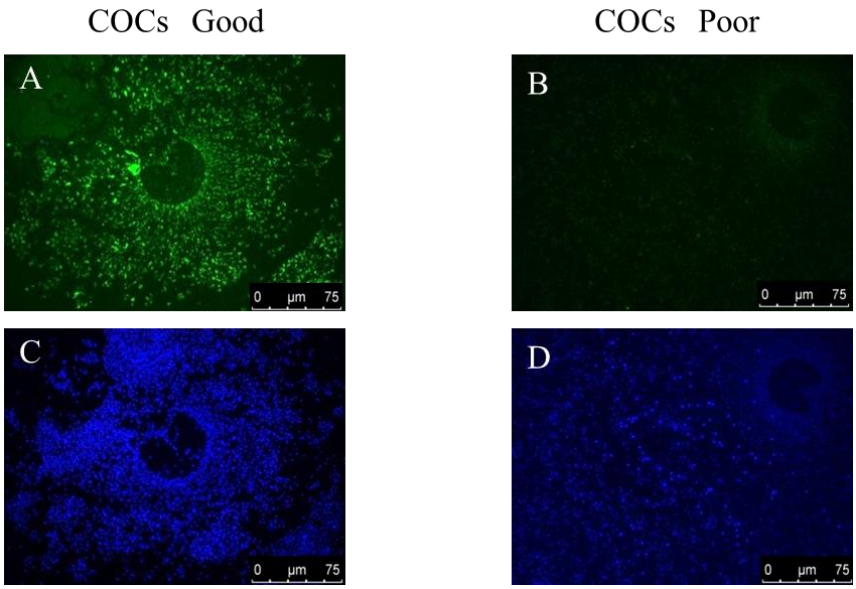

Fig. 5. Autophagic activity in good (A) and poor (B) COCs. The corresponding DNA of good (C) and poor (D) COCs was labelled by Hoechst 33342. Autophagic activity and particles were barely detectable in the poor quality (B) oocytes, but strong fluorescence and larger particles in the good quality (A) oocytes. The scale bar represents 75 µm.

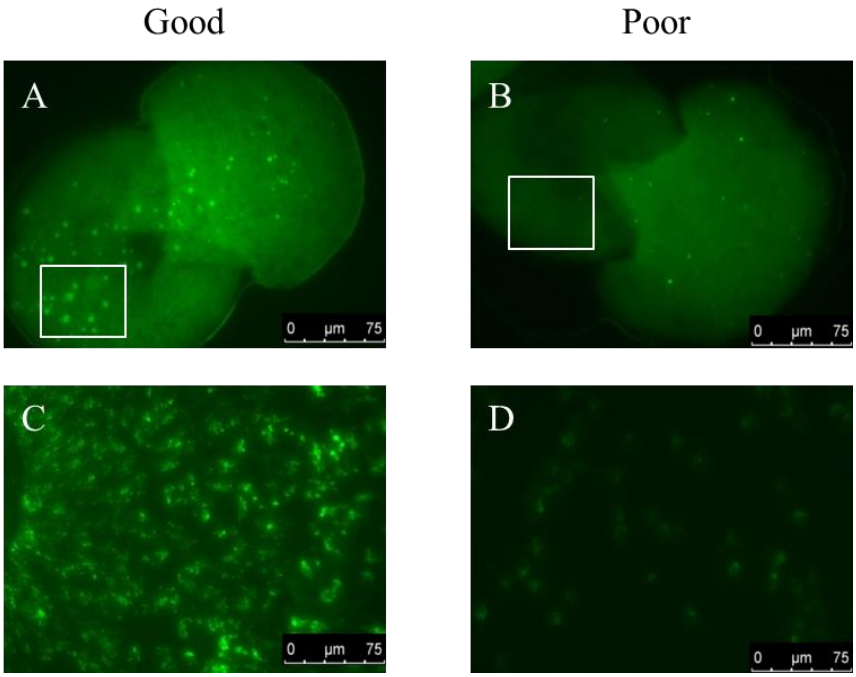

Fig. 6. Autophagic activity in good (A) and poor (B) quality oocyte and their cropped enlarged image (C, D). Autophagic activity (B) and particles (D) were barely detectable in the poor quality oocytes, but strong fluorescence (A) and larger particles (C) in the good quality oocytes. The scale bar represents 75 μm.

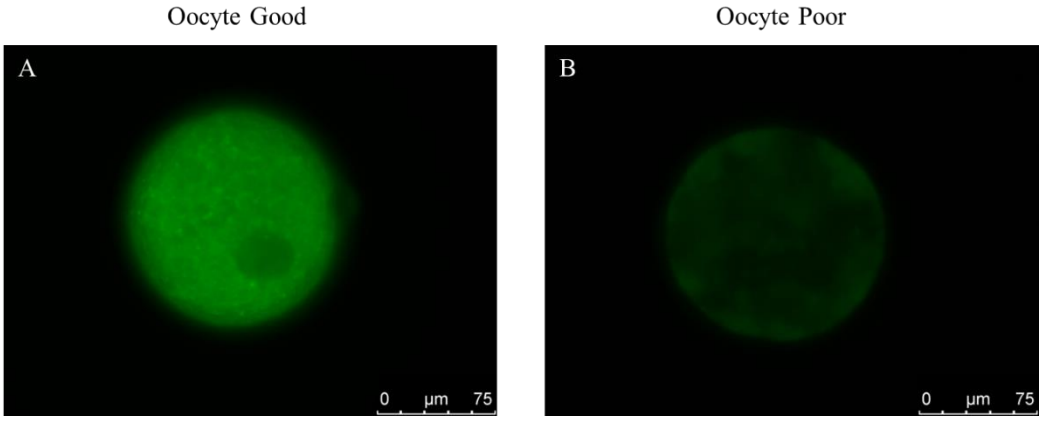

Fig. 7. Autophagic activity in bovine (germinal vesicle) GV stage oocytes of good and poor quality. Autophagic activity and particles were barely detectable in the poor quality (B) oocytes, but strong fluorescence and larger particles in the good quality oocytes (A). The scale bar represents 75 µm.

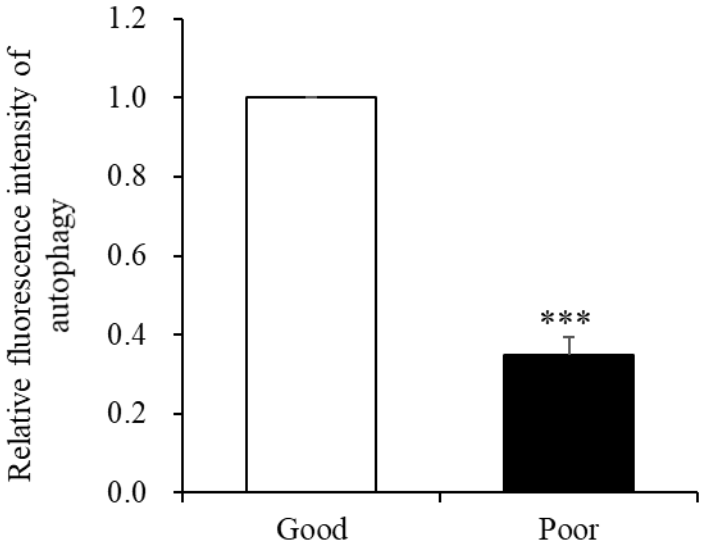

Fig. 8. Relative fluorescence intensity of autophagy (representing autophagic activity) between good and poor quality oocytes. The experiment was repeated three times. All data are shown as mean ± SEM. Asterisks indicate statistical difference (***$P < 0.001$) between good and poor quality oocytes.

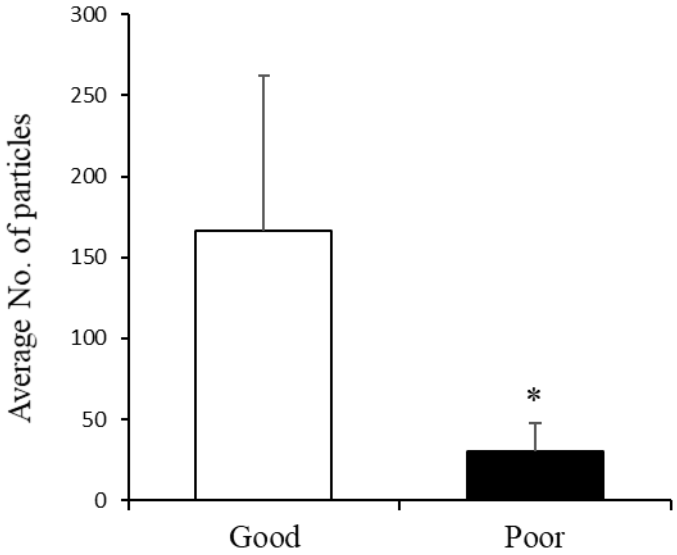

Fig. 9. Number of average particles (representing autophagosomes) between good and poor quality oocytes. The experiment was repeated thrice. All data are shown as mean ± SEM. Asterisks indicate statistical difference (*$P < 0.05$) between good and poor quality oocytes.

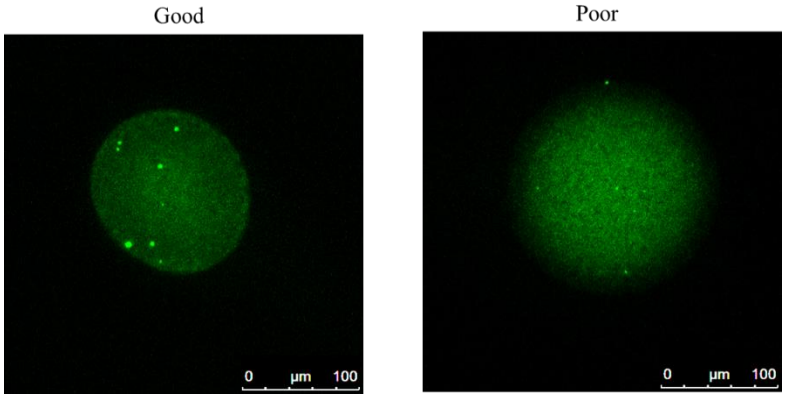

Fig. 10. Immunostaining of LC3 on good and poor quality oocyte. Larger number of LC3-stained particles were detected in good quality oocytes, but rarely observed in poor quality oocytes. The scale bar represents 100 μm.

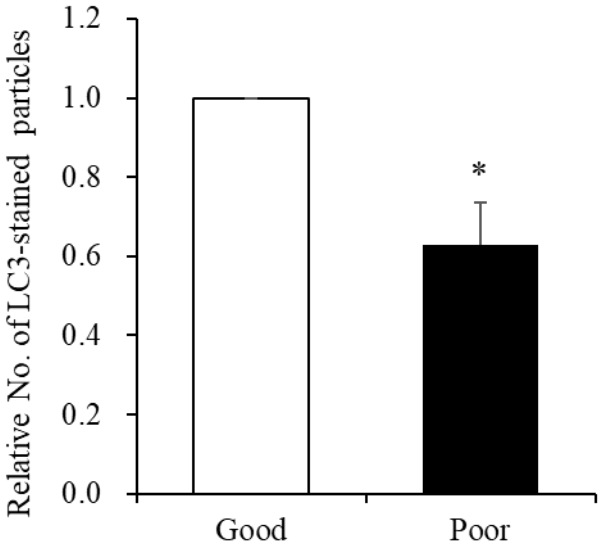

Fig. 11. Number of LC-stained particles (representing autophagosomes) in good and poor quality oocytes. The experiment was repeated thrice. All data are shown as mean ± SEM. Asterisks indicate statistical difference (*$P < 0.05$) between good and poor quality oocytes.

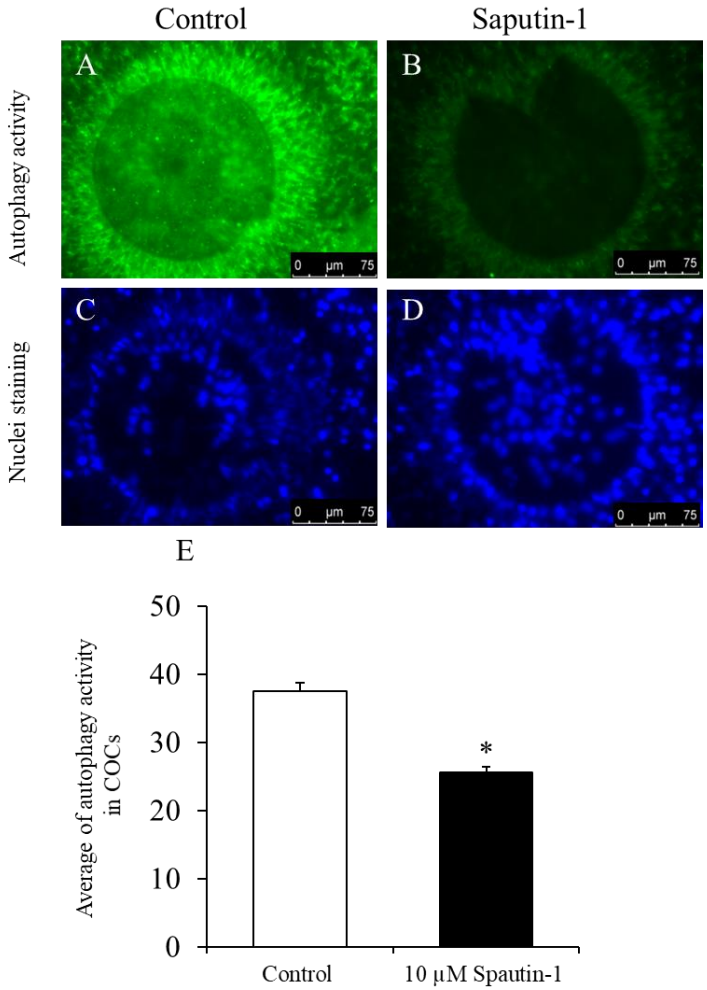

Fig. 12. Effect of autophagy inhibition on autophagic activity in COCs during IVM. Autophagic activity was higher in COCs without Saputin-1 (A) than that with Spautin-1 (B) during IVM. The corresponding DNA was labelled by Hoechst 33342 (C and D). Fluorescence intensity (representing autophagy activity) of COCs cultured with or without Spautin-1 (E). The scale bar represents 75 μm. This experiment was replicated three times. The data are expressed as the means ± SEM.; * $P < 0.05$.

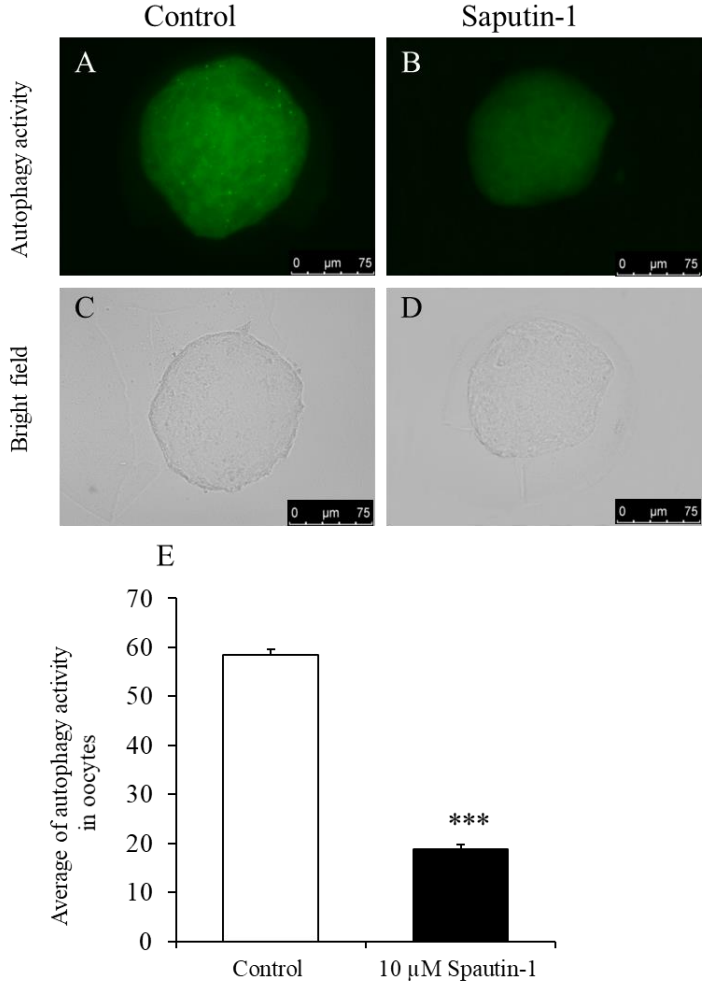

Fig. 13. Effect of autophagy inhibition on autophagic activity in oocytes during IVM. Autophagic activity was higher in COCs without Saputin-1 (A) than that with Spautin-1 (B) during IVM. The corresponding bright field image (C and D). Fluorescence intensity (representing autophagy activity) of COCs cultured with or without Spautin-1 (E). The scale bar represents 75 μm. This experiment was replicated three times. The data are expressed as the means ± SEM.; *** $P < 0.001$.

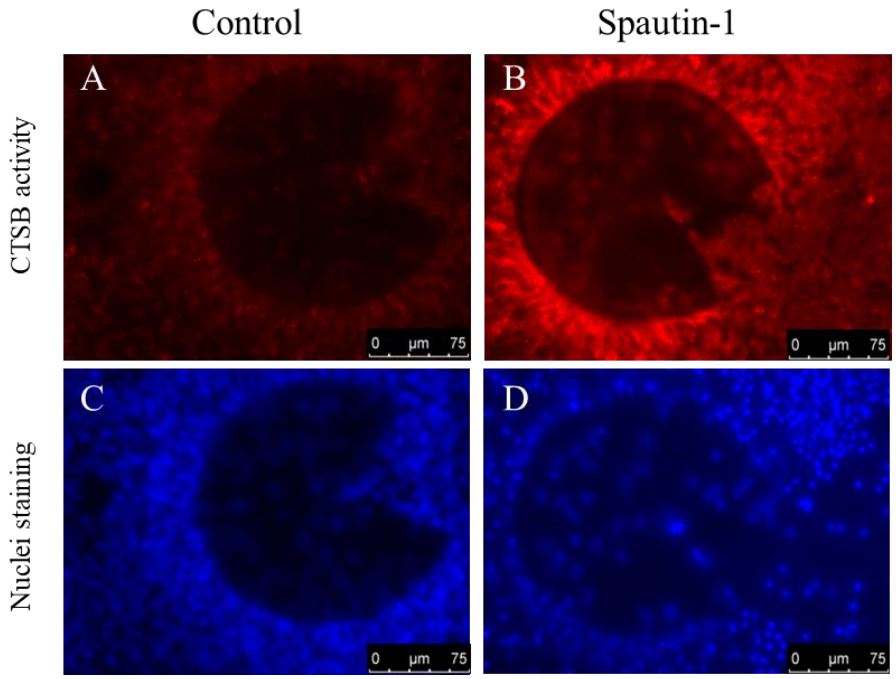

Fig. 14. Effect of autophagy inhibition on CTSB activity in COCs during IVM. CTSB activity was higher in COCs with Saputin-1 (A) than that without Spautin-1 (B) during IVM. The corresponding DNA was labelled by Hoechst 33342 (D and C). The scale bar represents 75 µm.

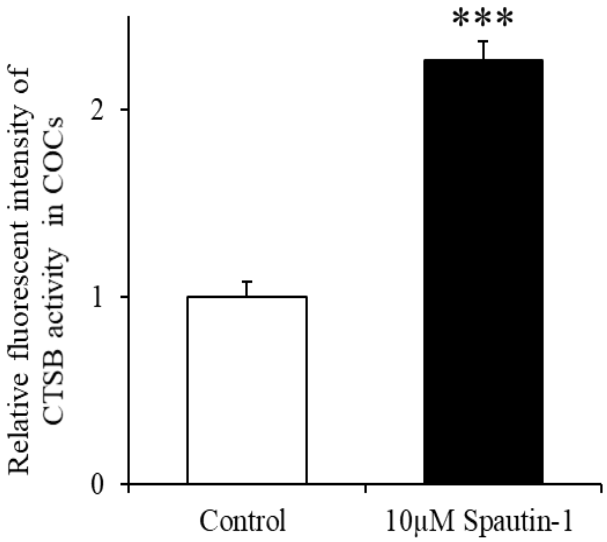

Fig. 15. Effect of autophagy inhibition on CTSB activity in COCs during IVM. Fluorescence intensity (representing CTSB activity) of COCs cultured with or without Spautin-1 during IVM. This experiment was replicated three times. The data are expressed as the means ± SEM.; *** $P < 0.001$.

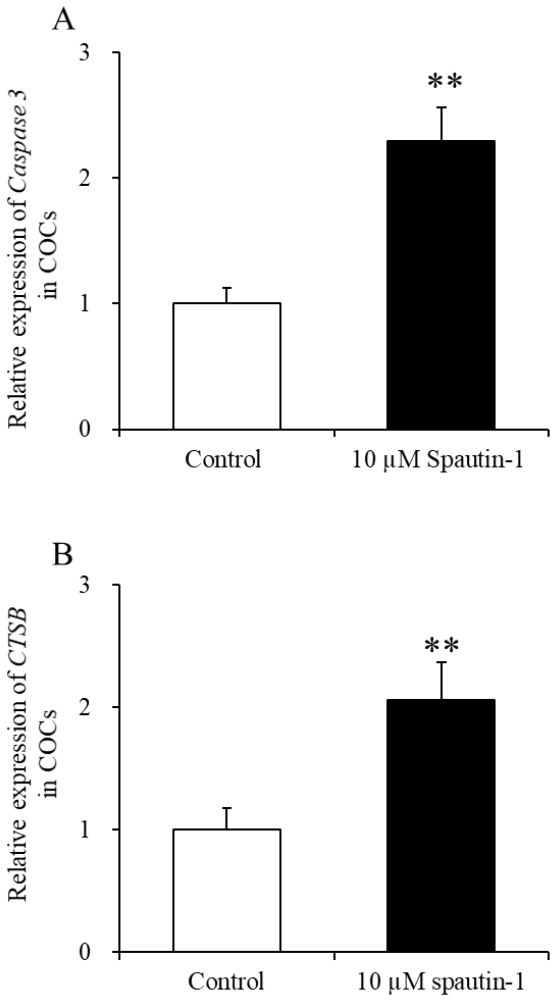

Fig. 16. Effect of autophagy inhibition on *CTSB* and *Caspase3* expressions in COCs during IVM. Spautin-1, an autophagy inhibitor was added into IVM medium for 22 h. The mRNA levels of *caspase 3* (apoptosis-related gene) (A) and *CTSB* (B) was significantly increased in the COCs during IVM with spautin-1 compared to the control group. This experiment was replicated three times. The data are expressed as the means ± SEM.; ** $P < 0.01$.

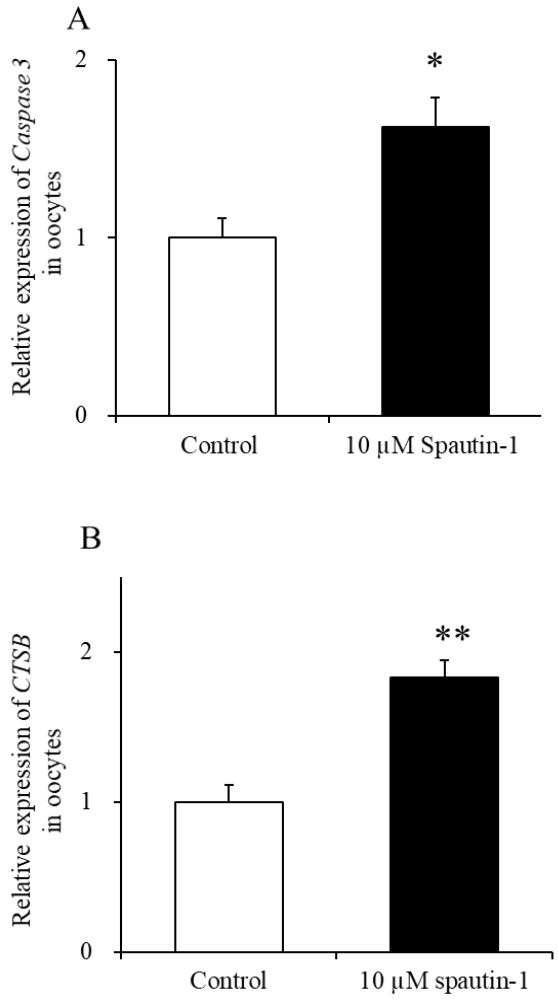

Fig. 17. Effect of autophagy inhibition on *CTSB* and *Caspase 3* expressions in oocytes during IVM. Spautin-1, an autophagy inhibitor was added into IVM medium for 22 h. The mRNA levels of *caspase 3* (apoptosis-related gene) (A) and *CTSB* (B) was significantly increased in the oocytes during IVM with spautin-1 compared to the non-treatment group. This experiment was replicated three times. The data are expressed as the means ± SEM.; * $P < 0.05$, ** $P < 0.01$.

Table 1. Effect of autophagy inhibition by Spautin-1 (10 μM) on developmental competence of bovine oocytes during IVM

Treatment	No. of replication	No. of inseminated oocytes	Cleavage rate (%)	Blastocyst rate (%)	Total cell number
Control	6	242	73.0±2.6	33.1±2.9	134.3±3.3
Spautin-1	6	251	51.0±2.5***	22.6±1.4*	109.8±2.7**

Data are represented as the mean ± standard error of six replicates.

* $P < 0.05$ vs. control, ** $P < 0.01$ vs. control，*** $P < 0.001$ vs. control.

Discussion

In this chapter, bovine COCs collected from ovaries were classified as good and poor quality according to their morphology. The good and poor COCs after IVM were subjected to IVF and IVC for 8 days. It was confirmed that lower total cell number and higher TUNEL positive cells of blastocysts derived from poor quality COCs than those from good quality of COCs and oocytes. Then, autophagic activity was detected with fluorescent dye both in poor and good COCs and oocytes. Autophagic activity was significantly higher in good quality oocytes than in poor quality ones. Immunostaining of LC3 representing the autophagosomes also revealed higher number of LC3-stained particles in good quality of oocytes than poor.

It is well known that the good quality oocytes after fertilization contain preferable development competence compared to poor quality ones. In general, embryo quality is evaluated by their morphology [1]; However, this parameter dose not correlate with embryo quality perfectly. The total cell number and the apoptosis cell rate of the blastocysts are the powerful tools to judge the quality of embryos [69]. In this study, I firstly confirmed that lower total cell number and high TUNEL positive cells of blastocysts derived from poor quality COCs than from good quality of COCs.

Recent studies have revealed that higher CTSB activity was observed in poor quality bovine and porcine GV oocytes and inhibition of CTSB activity by E-64 (1 μM) decreased its activity and improved embryonic developmental competence [41,62]. However, autophagic activity in good and poor quality oocytes in cattle remains unclear.

This study investigated autophagic activity and LC3-stained particles in COCs and oocytes. The higher autophagic activity and larger number of LC3-stained fluorescent particles detected in good quality COCs and GV stage oocytes is consistent with the previous findings that a higher autophagy activity in poor quality mouse embryos was observed [15]. Moreover, previous investigations showing the higher autophagic activity (good quality) embryos showed the excellent developmental competence and subsequent full-term embryonic development compared to that of lower ones (poor quality), suggesting that autophagy activity is related to the ability of an embryo to undergo normal development [15]. However, previous research has revealed that the GFP-LC3 dots were rarely detected in mouse MII oocytes and the LC3 conversion was increased in two-cell embryos relative to MII oocytes [14], which is different from my results. One possibility of this discrepancy might be due to the difference in species (between mouse and bovine). On the other hand, the MII oocytes were collected *in vivo* during ovulation in mouse; however, in present study, the COCs and GV stage oocytes were collected from the small follicles *in vitro*. Therefore, it is necessary to compare the quality of oocytes that were selectively ovulated and non-selectively collected followed by IVM.

In the present study, inhibitory effect of autophagy during oocytes maturation resulted in decreasing effect on oocyte autophagic activity and embryonic developmental competence, which revealed that autophagy is necessary during oocyte maturation and normal embryonic development. These results are consistent with recent report

revealing that inhibition of autophagic activity during oocyte maturation impaired the further developmental potential of porcine oocytes [70]. Taken together, these findings suggested that autophagy is not only essential for fertilization but also necessary for oocyte maturation and subsequent embryonic development.

It has been investigated that CTSB activates caspase indirectly by inducing mitochondrial membrane degradation, resulting in the translocation of apoptosis-inducing components from the mitochondria to the cytoplasm [56]. Indeed, inhibition of CTSB activity decreased apoptosis by preventing cytochrome c release from mitochondria in porcine parthenotes [62]. Moreover, recent researches have revealed that higher CTSB expression and activity were detected in poor quality bovine and porcine embryos [41,62]. Importantly, inhibition of CTSB activity increased the developmental competence of both bovine and porcine embryonic development [41,62].

To investigate the role of autophagy in oocyte quality, autophagic inhibition is one of the ways. There are many autophagy inhibitors to affect autophagy signal pathways like 3-MA and spautin-1. In present study, spautin-1 was chosen to inhibit autophagy, because its inhibitory mechanism has been well investigated [68]. Spautin-1 can promote the degradation of phosphatidylinositol phosphate 3 kinase (PI3PK) complexes by inhibiting two ubiquitin-specific peptidases, USP10 and USP 13, which target to the Beclin 1 subunit of PI3K complexes [68]. Among autophagy mediators, Beclin 1 plays an important role in autophagy signal pathways, as it interacts with many factors which regulate PI3P kinase which is crucial for activating autophagy [71,72]. Spautin-1 can

inhibit the function of Beclin 1, thus autophagosomes cannot be formed, thereby inhibiting autophagy process.

In the present study, an interrelation between autophagy and apoptosis in respect of oocyte maturation has been established. Inhibition of autophagy during IVM significantly increased CTSB activity, gene expression levels of *CTSB* and *caspase 3* in IVM oocytes. Inhibition of autophagy increased CTSB activity in good quality oocytes which is supported by Balboula et, at. who found that CTSB activity was higher in poor quality bovine oocytes [41]. These findings suggested CTSB is negatively correlated with bovine oocyte quality and can be considered as a mediator of apoptosis.

Taken together, these results suggest that negative relation between the quality and developmental competence, and autophagic activity associated with CTSB in bovine oocytes.

Chapter 3 **Effect of regulation of autophagy and CTSB on the developmental competence of good and poor quality bovine oocytes**

Introduction

Autophagy is a pivotal process during mammalian embryo development. It has been revealed that lack of autophagy results in development arrest, delay or deficiencies of development [73]. Autophagy is highly induced after fertilization [14], and induction of autophagy by its inducer rapamycin promoted preimplantation development of bovine embryos and increased porcine parthenotes development by decreasing apoptosis, as well as restored autophagy level and increased cloned mouse embryo viability [74–76].

On the other hand, inhibition of CTSB activity increased the developmental competence of both bovine and porcine preimplantation embryos [41,62]. Moreover, higher CTSB activity was observed in poor quality and heat-shocked bovine oocytes than in controls, and inhibition of such activity increased the developmental rate and the quality of produced embryos after IVF [41,63]. This indicates that lysosomal CTSB regulation is a promising strategy to improve the quality of embryos produced *in vitro*.

However, the combined effect of autophagy induction and CTSB inhibition on bovine preimplantation embryo development and quality is still unknown, especially for rescuing poor quality oocytes. Therefore, the negative correlation between autophagy

and CTSB led me to investigate the effect of CTSB inhibition and autophagy induction during IVM on the developmental competence of bovine embryos derived from poor quality oocytes.

Materials and methods

Oocyte collection and IVM

Described previously (Chapter 2), materials and methods section.

IVF and IVC

Described previously (Chapter 2), materials and methods section.

RNA extraction and quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA of twenty MII oocytes or five COCs after IVM was isolated using a ReliaPrep RNA Cell Miniprep System (Promega) according to the manufacturer's instructions. The extracted RNA was immediately used for RT-PCR or stored at −80˚C until analysis. After standardizing the RNA quantity using a NanoDrop spectrophotometer (Thermo Fisher Scientific), cDNA was then synthesized using the ReverTra, Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). On the other hand, cDNA from small number of oocytes (one or two) was synthesized with a Super Prep^{TM} Cell Lysis & RT Kit for qPCR (Toyobo) kit. Finally, qRT-PCR was performed to evaluate the expression of *LC3α* and *LC3β* transcripts relative to histone *H2AFZ* using Light Cycler® 480 (Roche). The primers used for the analysis are described in (Supplemental list, Table. 1). The reactions were carried out in 96-well PCR plates, in a total volume of 10 µl containing 5 µl of Thunderbird SYBR qPCR Mix (Toyobo), 1 µl of each primer

(10 pmol/μl), and 3μl of cDNA, and then subjected to the following cycling conditions: one cycle at 95˚C for 30 sec (denaturation), followed by 55 cycles at 95˚C for 10 sec (amplification), 55˚C for 15 sec (primer annealing), and 72˚C for 30 sec (extension), a melting-curve step at a gradient of 55–95 ˚C with the increment of 2.2 ˚C/sec, and a cooling step at 4 ˚C. The relative expression levels of each of the target genes were calculated relative to that of internal control gene, histone family, membrane Z (*H2AFZ*).

Detection of autophagic activity in E-64, rapamycin and E-64 + rapamycin-treated poor quality metaphase II (MII) oocytes

Autophagic activity in E-64, rapamycin, E-64 + rapamycin-treated MII oocytes was examined using Cyto-ID Green Autophagy Detection Reagent (Enzo Life Sciences), an excellent amphiphilic autophagosome tracer dye that measures the autophagic vacuoles and monitors autophagic flux in live cells [20], according to the manufacturer's protocol with some modifications. Briefly, after removing surrounding cumulus cells MII oocytes were incubated in 500 μl 1× Assay Buffer (Enzo) including 1 μl/ml reaction mix at 38.5 °C in a humidified atmosphere of 5% CO_2 and 5% O_2 in air for 45 min. Nuclei were stained with 25 μg/ml Hoechst 33342 (Sigma-Aldrich) prepared in reaction medium. After rinsing thrice in 0.2% PVA-PBS, the oocytes were mounted onto a glass slide and observed under a fluorescence microscope using a 550-nm filter (LAS X, Leica).

Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assay

Described previously (Chapter 2), materials and methods section.

Experimental design

After collection, COCs were divided into two groups based on their morphology (as described in chapter 2) and the further experiments were performed as follows:

Experiment 1: Effect of autophagy induction, CTSB inhibition, and combined administration on autophagic activity of poor quality oocytes during IVM.

To evaluate whether autophagy induction, CTSB inhibition, and their combination treatment can induce autophagy activity of poor quality oocytes during IVM, 1 μM of E-64, 100 nM of rapamycin (an autophagy inducer) and 1 μM of E-64 + 100 nM of rapamycin were supplemented into IVM medium. After maturation, the denuded oocytes derived from control (non-reagent treatment) and each indicated treated group were subjected to examine autophagic activity.

Experiment 2: Effect of CTSB inhibition on autophagy related genes expression during IVM

Considering CTSB inhibition by E-64 increased autophagy activity, I investigated the relationship between CTSB inhibition and gene expression level of autophagy-related genes (*LC3α* and *LC3β*) in COCs and MII oocytes during IVM. COCs were *in vitro* matured with or without 1 μM E-64. The gene expression levels of *LC3α* and *LC3β* of

COCs and denuded MII oocytes were examined by qRT-PCR.

Experiment 3: Effect of autophagy induction, CTSB inhibition and combined administration of E-64 and rapamycin on embryo developmental competence and quality during IVM.

Good or poor quality COCs underwent IVM and were each divided into four groups as follows: 1) TCM-199 alone (control group), 2) TCM-199 supplemented with 1 μM E-64, 3) TCM-199 supplemented with 100 nM rapamycin, and 4) TCM-199 supplemented with 1 μM E-64 + 100 nM rapamycin. After IVF and IVC, cleavage and blastocyst rates were assessed on day 2 and 8, respectively. Total cell number and apoptotic index were evaluated on day 8 embryos.

Statistical analysis

Data are representative of at least three or five independent experiments. All data are shown as the mean ± standard error of the mean (SEM). Statistically significant differences were assessed by Student's *t-test* and one-way analysis of variance (ANOVA)-Tukey's Multiple Range Test implemented in Graphpad Prism® 7 Software (La Jolla, CA, USA). All percentage data were arcsine transformed prior to statistical analysis. *P* values < 0.001, < 0.01, or < 0.05 were considered statistically significant. *P* values < 0.1 were regarded as indicating a tendency.

Results

Effect of E-64, rapamycin, and E-64 + rapamycin treatment on autophagic activity of poor quality MII oocytes

Considering that autophagic activity is lower in poor quality COCs (Fig. 5) and GV stage oocytes (Fig. 6 and 7), I tried to elucidate whether E-64, rapamycin, and E-64 + rapamycin can induce autophagy in poor quality oocytes. Poor quality COCs were matured with or without 1 μM E-64, 100 nM rapamycin, and 1 μM E-64 + 100 nM rapamycin. After maturation, MII oocytes were denuded prior to evaluating autophagic activity. Autophagic activity was clearly observed in the cytoplasm of all oocytes (Fig. 18). Importantly, E-64 ($P < 0.05$), rapamycin ($P < 0.01$) as well as E-64 + rapamycin ($P < 0.01$) significantly increased the fluorescence intensity of autophagic activity when compared to that in the control (Fig. 19), indicating that E-64 and rapamycin can induce autophagic activity in bovine IVM system.

Effect of CTSB inhibition on *LC3α* and *LC3β* gene expression in COCs and oocytes during IVM

The effect of CTSB inhibition on gene expression of autophagy-related genes, (*LC3α* and *LC3β*) in COCs and oocytes after IVM were examined by qRT-PCR. After cultured with 1μM of E-64 into IVM medium, gene expression levels of *LC3α* and *LC3β* were significantly higher in both COCs (Fig. 20) and oocytes (Fig. 21) compared to non-treatment group.

Effect of E-64 and rapamycin on the developmental competence of good and poor quality COCs

To investigate the effect of CTSB inhibition and autophagy induction on the developmental competence of good and poor quality COCs, 1 μM of E-64 and/or 100 nM of rapamycin was added to IVM medium (TCM-199). E-64 is a cell permeable inhibitor of cysteine proteases, whose efficiency to inhibit CTSB was validated in bovine oocytes [41]. Supplementation of E-64 and/or rapamycin during IVM improved the morphological quality of day 8 blastocysts compared to control group (Fig. 22 and 23). Addition of rapamycin alone and E-64 + rapamycin treatment groups improved embryonic development especially derived from poor quality COCs. In poor quality COCs, supplementation of rapamycin alone (73.4 ± 4.6) or E-64 + rapamycin (69.3 ± 4.7) significantly increased cleavage rates when compared to that in the control group (46.7 ± 7.6, $P < 0.05$). Supplementation of IVM medium with E-64 + rapamycin significantly ($P < 0.05$) increased the blastocyst rate (30.2 ± 6.3), and rapamycin alone showed a high increasing tendency (29.4 ± 5.2) when compared to that in the control group (10.8 ± 2.4; Table 2.). Even though there was no significant difference in cleavage rate between the control group and every other E-64 and/or rapamycin treated group, E-64 (42.1 ± 3.1) alone significantly ($P < 0.05$) increased the blastocyst rate and E-64 + rapamycin (46.3 ± 4.4) showed an increasing tendency compared to that in the control group (31.5 ± 2.4) derived from good quality COCs (Table 3). Importantly, addition of rapamycin alone (29.4 ± 5.2) or E-64 + rapamycin (30.2 ± 6.3) during IVM of poor

quality COCs improved blastocyst rate to a similar extent to that observed in the good quality COCs control group (31.5 ± 2.4; Tables 2 and 3).

Effect of E-64 and rapamycin on embryo quality in blastocyst stage and apoptotic level of COCs

Both E-64 or rapamycin alone and E-64 + rapamycin treatment improved the embryo morphology derived from poor quality COCs (Fig. 22). In addition, E-64 alone and E-64 + rapamycin treatment promoted the embryo quality derived from good quality COCs (Fig. 23). To investigate the quality of blastocysts produced from good and poor quality COCs, total cell number and TUNEL-positive cell number were evaluated (Fig. 24A and 24B). In poor quality COCs-derived embryos, supplementation of E-64 or rapamycin alone ($P < 0.05$), and E-64 + rapamycin ($P < 0.01$) significantly increased the total cell number of blastocysts compared to that in the control group (Fig. 25), which was supported by the E-64 or rapamycin alone ($P < 0.01$) and E-64 + rapamycin ($P < 0.001$) treatment significantly decreasing the apoptotic index compared to that in the control group (Fig. 27). In good quality COCs-derived embryos, E-64 alone ($P < 0.05$) and E-64 + rapamycin ($P < 0.01$) treatment groups significantly increased the total cell number of blastocysts compared to that in the control group (Fig. 26). This result is supported by the apoptotic index that was significantly decreased in the E-64 alone ($P < 0.01$) and in the E-64 + rapamycin group ($P < 0.001$) compared to that in the control group (Fig. 28). In addition, E-64 + rapamycin treatment significantly decreased the

apoptotic index compared to that in the rapamycin alone group ($P < 0.05$) (Fig. 28).

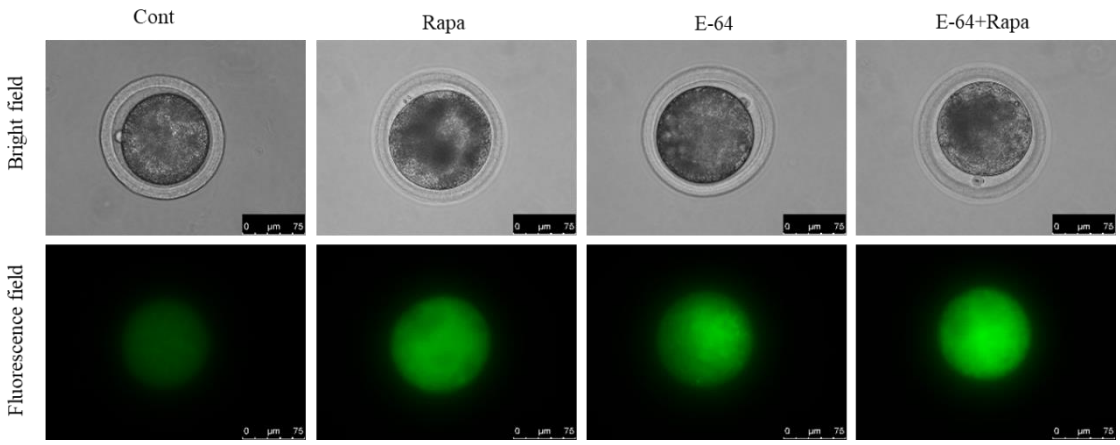

Fig. 18. Effect of E-64, rapamycin, and E-64 + rapamycin treatment on autophagy activity in poor quality MII oocytes during IVM. Representative images of autophagic activity of bright field images and corresponding fluorescent images of poor quality MII oocytes after IVM of the indicated treatment groups (control group: Cont; E-64 group: E-64; rapamycin group: Rapa; E-64 + rapamycin group: E-64 + Rapa). The experiment was repeated three times. The scale bar represents 75 μm.

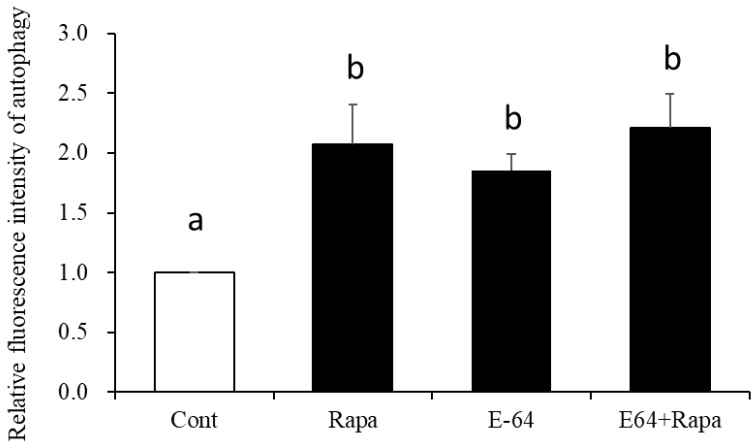

Fig. 19. Effect of E-64, rapamycin, and E-64 + rapamycin treatment on autophagy activity during IVM poor quality MII oocytes. Relative fluorescence intensity of autophagy (representing autophagic activity) between the indicated treatment groups (control group: Cont; E-64 group: E-64; rapamycin group: Rapa; E-64 + rapamycin group: E-64 + Rapa). The experiment was repeated thrice. All data are shown as mean ± SEM. Bars with different letters indicate statistical significance (a vs. b：$P < 0.05$).

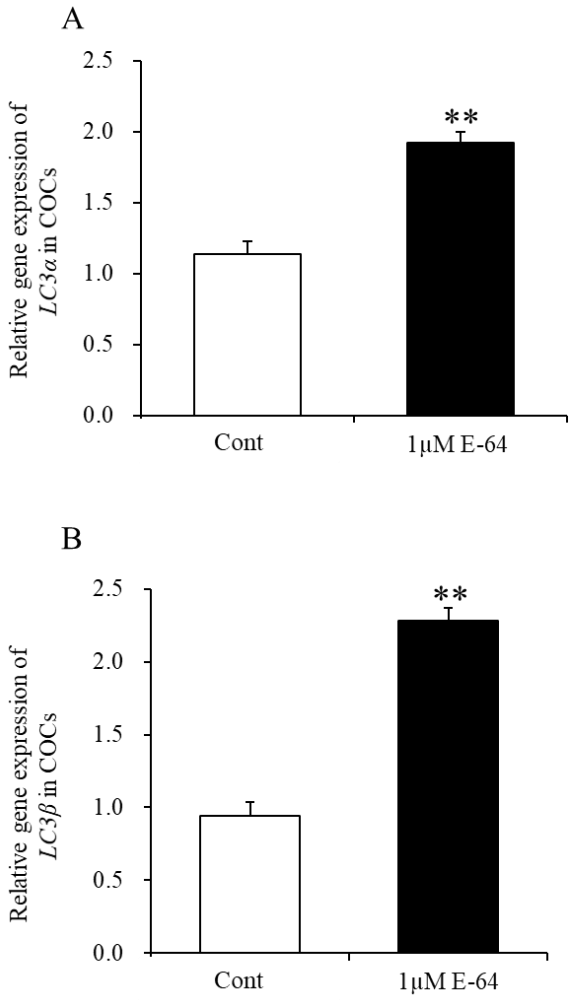

Fig. 20. Effect of CTSB inhibition on *LC3α* and *LC3β* expressions in COCs during IVM. E-64, an CTSB inhibitor was added into IVM medium for 22 h. The mRNA levels of *LC3α* (A) and *LC3β* (B) was significantly increased in the COCs during IVM with E-64 compared to the non-treatment group. This experiment was replicated three times. The data are expressed as the means ± SEM.; ** $P < 0.01$. Cont: control.

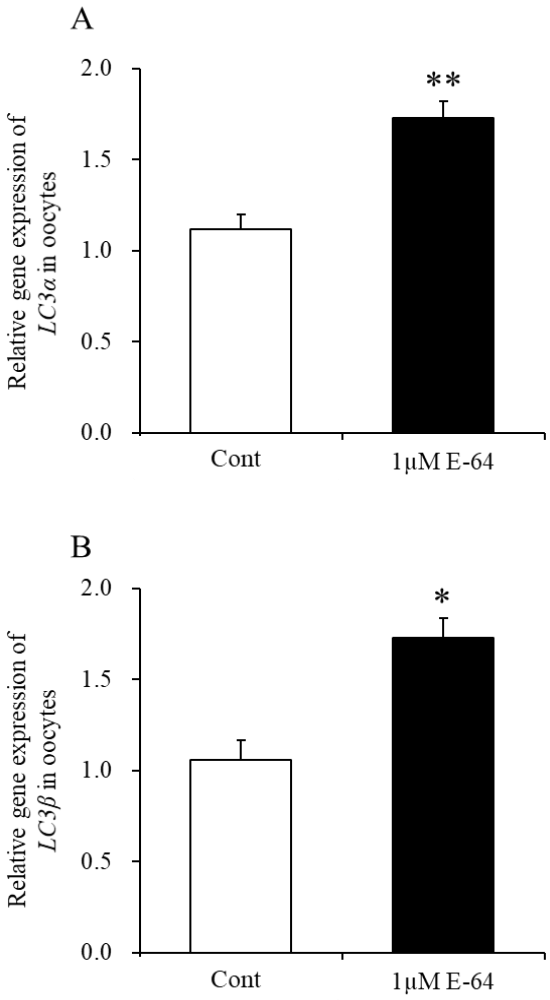

Fig. 21. Effect of CTSB inhibition on *LC3α* and *LC3β* expressions in oocytes during IVM. E-64, an CTSB inhibitor was added into IVM medium for 22 h. The mRNA levels of *LC3α* (A) and *LC3β* (B) was significantly increased in the oocytes during IVM with E-64 compared to the non-treatment group. This experiment was replicated three times. The data are expressed as the means ± SEM.; * $P < 0.05$, ** $P < 0.01$. Cont: control.

Table 2. Effect of E-64 (1 μM) and/or rapamycin (100 nM) treatment during IVM on subsequent cleavage and blastocyst rates in poor quality COCs derived bovine embryos

Treatment	No. of replication	No. of inseminated oocytes	Cleavage rate (%)	Blastocyst rate (%)
Cont	5	119	46.7 ± 7.6	10.8 ± 2.4
E-64	5	107	67.0 ± 4.6	19.2 ± 4.4
Rapa	5	111	73.4 ± 4.6*	29.4 ± 5.2#
E-64+Rapa	5	112	69.3 ± 4.7*	30.2 ± 6.3#

Data are represented as the mean ± standard error of five replicates.

*: $P < 0.05$ vs. Cont, #: $P < 0.1$ vs. Cont

Cont: control; Rapa: rapamycin; E-64 + Rapa: E-64 + rapamycin

Table 3. Effect of E-64 (1 μM) and/or rapamycin (100 nM) treatment during (*in vitro* maturation) IVM on subsequent cleavage and blastocyst rates in good quality COCs derived bovine embryos

Treatment	No. of replication	No. of inseminated oocytes	Cleavage rate (%)	Blastocyst rate (%)
Cont	5	99	76.7 ± 3.9	31.5 ± 2.4
E-64	5	98	87.3 ± 4.3	42.1 ± 3.1*
Rapa	5	90	75.5 ± 3.7	34.3 ± 2.9
E-64+Rapa	5	100	84.6 ± 0.9	46.3 ± 4.4*

Data are represented as the mean ± standard error of five replicates.

*: $P < 0.05$ vs. Cont

Cont: control; Rapa: rapamycin; E-64 + Rapa: E-64 + rapamycin

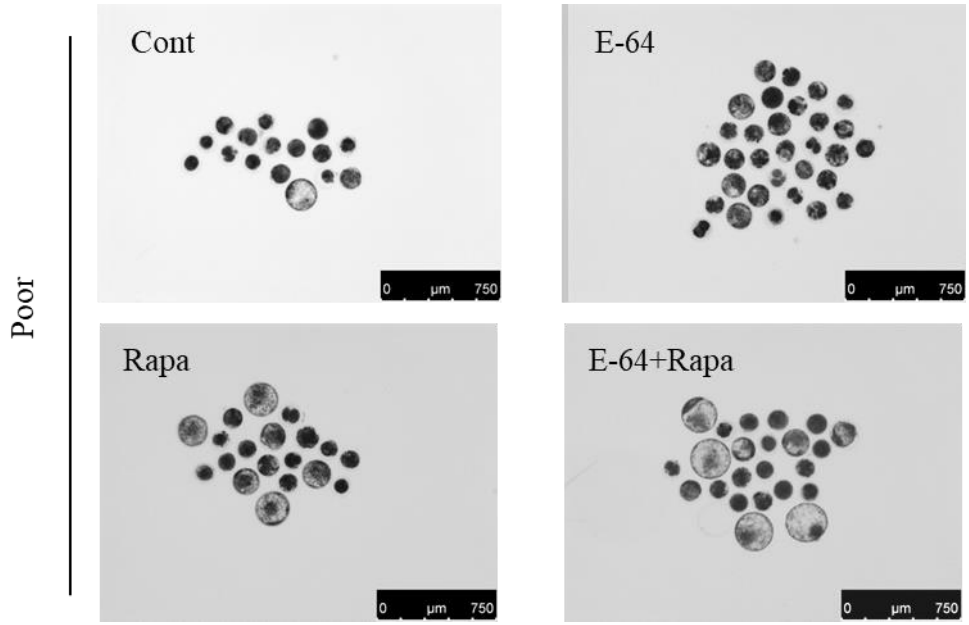

Fig. 22. Representative photographs of poor quality blastocysts in the indicated treatment groups (control group: Cont; E-64 group: E-64; rapamycin group: Rapa; E-64 + rapamycin group: E-64 + Rapa) derived from poor quality COCs, respectively. The scale bar represents 750 µm.

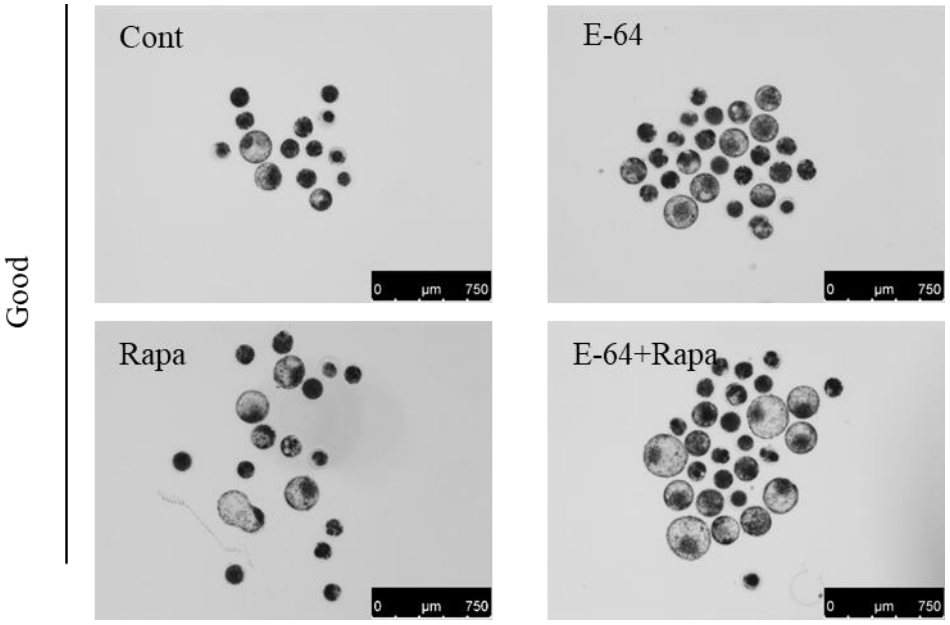

Fig. 23. Representative photographs of good quality blastocysts in the indicated treatment groups (control group: Cont; E-64 group: E-64; rapamycin group: Rapa; E-64 + rapamycin group: E-64 + Rapa) derived from good quality COCs, respectively. The scale bar represents 750 μm.

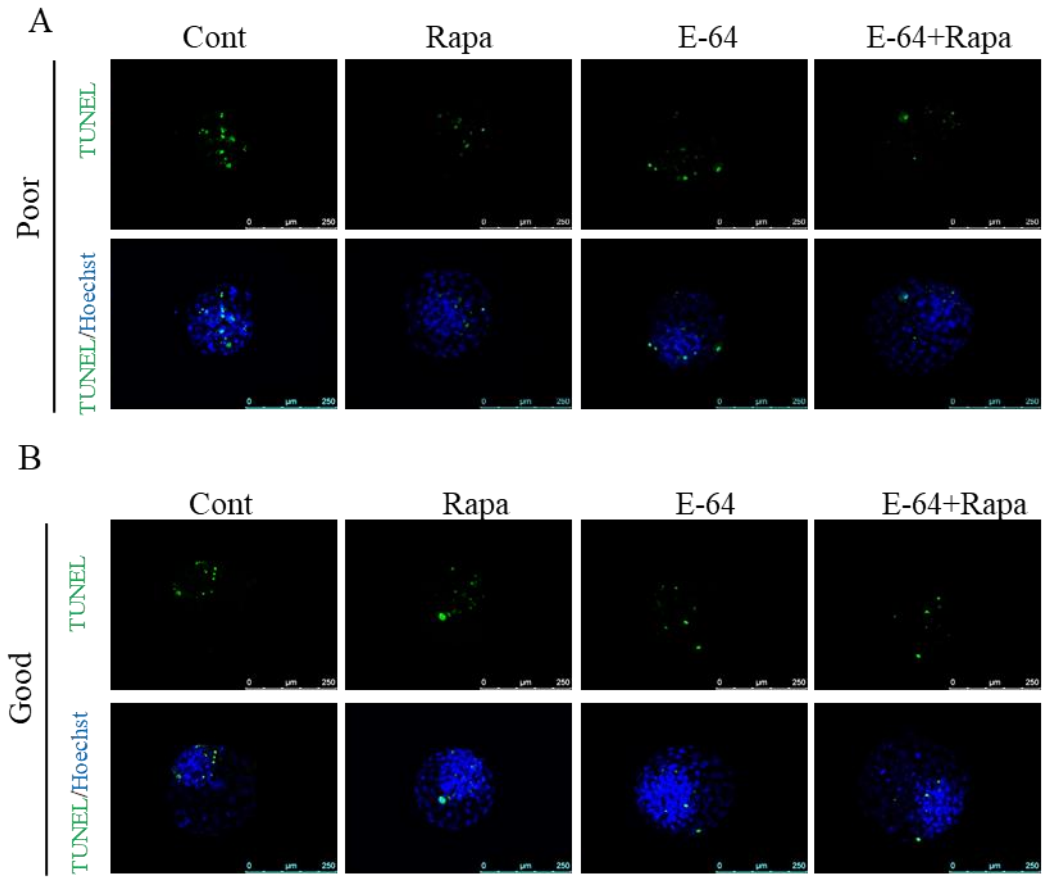

Fig. 24. Improvement of blastocyst quality through inhibition of CTSB activity and induction of autophagy during IVM of IVP poor and good quality embryos. Representative images of apoptosis detection analysis using poor (A) and good (B) quality blastocysts of the indicated treatment groups (control group: Cont; E-64 group: E-64; rapamycin group: Rapa; E-64 + rapamycin group: E-64 + Rapa). Light green dots show the TUNEL and blue color show the Hoechst 33342 for nuclei. The merged images are presented in green (TUNEL) and blue (Hoechst 33342). The scale bar represents 250 μm.

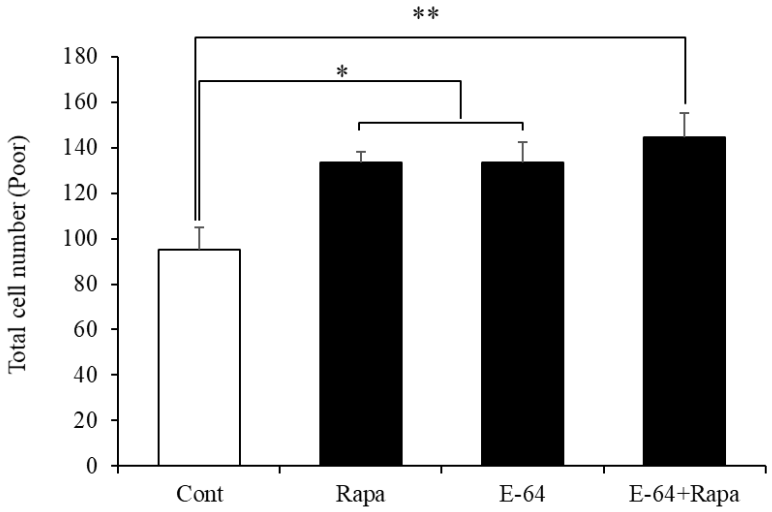

Fig. 25. Improvement of blastocyst quality through inhibition of CTSB activity and induction of autophagy during IVM of IVP poor quality embryos. Total cell number of each indicated group from poor quality blastocysts (control group: Cont; E-64 group: E-64; rapamycin group: Rapa; E-64 + rapamycin group: E-64 + Rapa). The experiment was repeated five times. All data are shown as mean ± SEM. Asterisks indicate statistical difference (*$P < 0.05$, **$P < 0.01$).

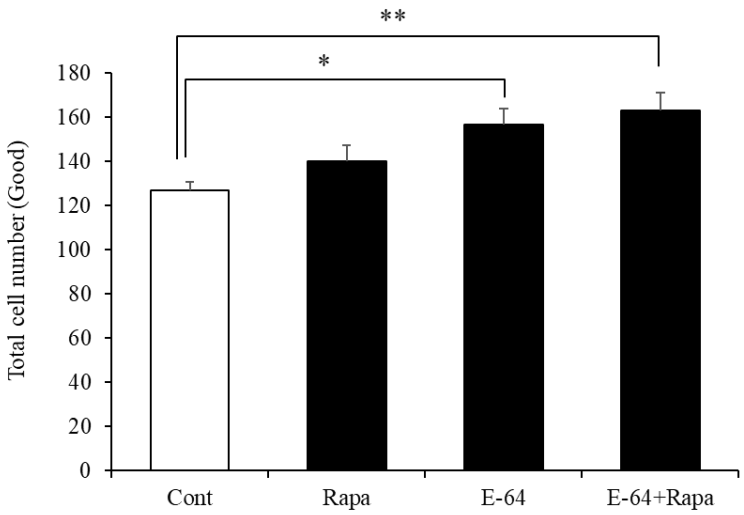

Fig. 26. Improvement of blastocyst quality through inhibition of CTSB activity and induction of autophagy during IVM of IVP good quality embryos. Total cell number of each indicated group from good quality blastocysts (control group: Cont; E-64 group: E-64; rapamycin group: Rapa; E-64 + rapamycin group: E-64 + Rapa). The experiment was repeated five times. All data are shown as mean ± SEM. Asterisks indicate statistical difference (*$P < 0.05$, **$P < 0.01$).

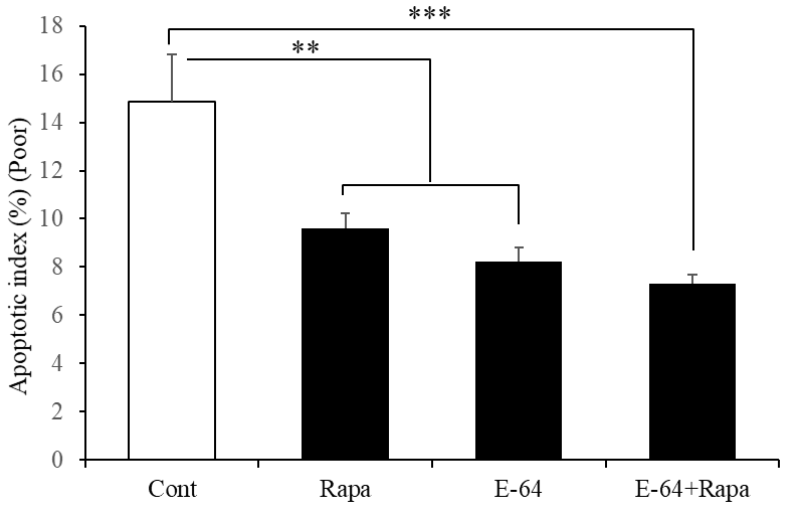

Fig. 27. Improvement of blastocyst quality through inhibition of CTSB activity and induction of autophagy during IVM of IVP poor quality embryos. Apoptotic index of each indicated group from poor quality blastocysts (control group: Cont; E-64 group: E-64; rapamycin group: Rapa; E-64 + rapamycin group: E-64 + Rapa). The experiment was repeated five times. All data are shown as mean ± SEM. Asterisks indicate statistical difference (**$P < 0.01$, ***$P < 0.001$).

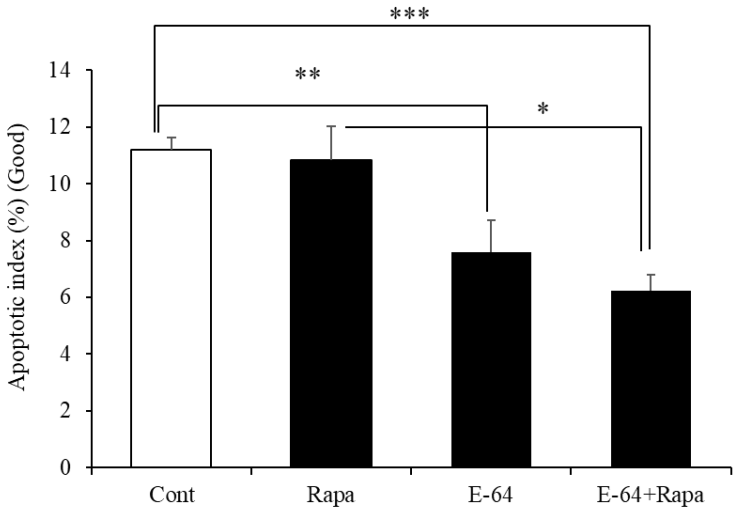

Fig. 28. Improvement of blastocyst quality through inhibition of CTSB activity and induction of autophagy during IVM of IVP good quality embryos. Apoptotic index of each indicated group from good quality blastocysts (control group: Cont; E-64 group: E-64; rapamycin group: Rapa; E-64 + rapamycin group: E-64 + Rapa). The experiment was repeated five times. All data are shown as mean ± SEM. Asterisks indicate statistical difference (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Discussion

Multiple factors, especially oocyte quality, affect the ability of the putative fertilized zygote to develop to the blastocyst stage, attachment to the maternal uterus, and to further ensure the health of the fetus [22]. Among them, follicular atresia plays a pivotal role in oogenesis and involves the removal of follicles not selected for ovulation. It is widely known that less than 1% of follicles ovulate, whereas the remaining over 99% of follicles undergo atresia in mammalian ovaries [23]; therefore, the number of oocytes of the highest quality available for IVM is very few. Lysosomal cathepsin-induced apoptosis is one of the mechanisms by which follicular atresia is induced in mammalians [77]. However, it has been demonstrated that autophagy altered follicular development, for example, the ovaries of autophagy-related gene 7 (*Atg7*) deficient mice contain fewer germ cells and primordial follicles [25,78]. Moreover, autophagy led to PCD in which regulation of follicular atresia was induced by oocytes [79].

In present study, increased autophagic activity after rapamycin treatment in poor quality MII oocytes is also partly supported by the increasing abundance of mRNA of *Beclin 1* and *LC3* in poor quality porcine MII oocytes after rapamycin treatment during IVM [80]. Interestingly, the present study clearly showed that E-64 treatment during IVM also increased autophagic activity, this result is supported by the previous research that lysosomal inhibitors like bafilomycin A_1 ($BafA_1$) and E64d activated early autophagy events like ATG16L1 and ATG12 puncta formation in mouse embryonic fibroblast cells and the induction effect of autophagy was similar to that of rapamycin

[81].

In addition, the processing of caspase zymogens and induction of nuclear apoptosis are inhibited by the synthetic peptide caspase inhibitors [56]. Caspases also cleave Beclin 1 in apoptosis pathway, thereby impairing its pro-autophagic activity [82]. Therefore, inhibition of CTSB results in accomplishing the autophagy formation through Beclin 1 mediated pathway. On the other hand, CTSB can activate the pro-apoptotic protein BID [83] that resulted in cytochrome c release and in turn subsequent caspase activation [84]. Moreover, knockdown of BID leaded to the suppression of apoptosis and shift to autophagy combined with the increasing of Beclin 1 and LC3 cellular content [85]. Thus, inhibition of CTSB resulted in inactivating of BID and increasing the autophagy level.

Taken together, these findings indicated that autophagy induction is regulated not only by rapamycin, but also by the inhibited CTSB mediated apoptosis pathway. Although in the present study, I just evaluate inhibition of CTSB by E-64 increased autophagy-related gene expression in good quality oocytes, but did not detect the autophagy activation by rapamycin / E-64 in good quality COCs, it is necessary to compare the autophagy activation mechanism in good and poor quality COCs.

Many parameters have been reported for evaluation of the quality of mammalian embryos, among which average total cell number and positive-TUNEL cell ratio are considered valuable tools for evaluating the quality of preimplantation embryos [86,87]. The validity of classification of good and poor quality COCs was confirmed by the total

cell number and apoptotic index of blastocysts derived from no treatment good and poor quality COCs (control group) considering the inverse relationship between CTSB and autophagic activity on oocyte quality. Subsequently, I investigated the effects of inhibition of CTSB and/or induction of autophagy during IVM on subsequent embryonic developmental competence and quality and apoptotic level of produced blastocysts by adding E-64 (1 μM) and/or rapamycin (100 nM) into IVM medium containing the COCs classified as good or poor quality according to their morphology. In the present study, supplementation of E-64 alone significantly increased the blastocyst rate (derived from good quality COCs) and improved the quality of blastocysts which was supported by the increased total cell number combined with the decreased apoptotic levels of blastocysts derived from both good and poor quality COCs. These results were consistent with the previous reports that addition of 1 μM E-64 during IVM decreased the CTSB activity and increased the developmental competence of good quality COCs with increase in the total cell number and decrease in the number of TUNEL-positive cells of blastocysts after IVM and IVC [41]. In addition, treatment of bovine and porcine embryos from IVC with 1 μM E-64 also decreased CTSB activity of their embryos, and increased developmental competence by decreasing the apoptotic index of blastocysts [63,64]. In contrast, with the addition of rapamycin alone, there was no difference in cleavage and blastocyst rate in day 2 and day 8 embryos and the total cell number of blastocysts did not change compared to that in the control blastocysts derived from good quality COCs. One possibility is that the

autophagic activity in good quality oocytes is essential for further development. Interestingly, in contrast to the good quality group, rapamycin treatment significantly increased the cleavage rate and showed the high tendency of blastocyst rate and increased the total cell number which was supported by the decreased TUNEL-positive cell number of blastocysts derived from poor quality COCs. Taken together, these results suggested that autophagy induction by rapamycin is more important of poor quality COCs for their further embryonic development than good quality COCs.

Present results were consistent with those of previous studies showing that rapamycin (10 nM) treatment of early stage embryos enhanced autophagy levels of IVP bovine, porcine, and somatic cell nuclear transfer produced mouse embryos and increased their subsequent embryo viability [74–76], and decreased the apoptosis levels of blastocysts [74,75]. Similarly, our results indicated that high concentration of rapamycin (100 nM) did not increase the total cell number or decrease the apoptotic index in good quality COCs but increased the total cell number and decreased the apoptotic index of blastocysts derived from poor quality COCs. This result is consistent with the previous reports that 1 nM rapamycin had no effect on blastocyst rate from good-COCs derived porcine embryos and 100 nM (except for 10 nM) of rapamycin did not increase the total cell number or decrease apoptotic levels [75,80]. Thus, taking into account that higher autophagic activity was observed in good quality oocytes and induction of autophagy using high concentration rapamycin did not improve the embryo quality, selection of a suitable concentration of rapamycin is important for autophagy induction to improve

embryo development quality derived from good (10 nM) and poor (100 nM) quality COCs. Although autophagy was originally discovered as a mechanism of cell survival, it has more recently been recognized to be involved in cell death [88]. Thus, over induction of autophagy by high does of rapamycin can also trigger apoptosis, indicating that high autophagic activity has a negative effect on blastocyst survival.

In addition, previous study has revealed that induction of autophagy increased TE cell number [75] by differential staining. The authors found that the ICM and TE cell numbers were significantly increased by 1 and 10 nM rapamycin and the TE ratio was markedly increased only by 10 nM rapamycin. However, in present study, I did not examine the TE ratio of blastocysts by differential staining, therefore it is necessary to evaluate the ICM and TE ratio in further experiments.

Finally, I also analyzed the effect of reduction in CTSB and induction of autophagy on embryo developmental competence with the supplementation of E-64 and rapamycin together into IVM medium. I found that E-64 + rapamycin treatment 1) significantly increased the cleavage rate of the embryos compared to that in the control group derived from poor quality COCs, 2) significantly increased the blastocyst rate compared to that in control blastocyst derived from poor quality COCs, or a high tendency of blastocyst rate compared to that in the control group derived from good quality COCs, respectively. 3) significantly increased the total cell number supported by decreasing apoptotic index of the blastocysts derived from both good and poor quality COCs, and 4) significantly decreased the apoptosis levels in blastocysts derived from good quality COCs compared

to the rapamycin alone treatment group. It was reported that inhibition of lysosomal proteases using $BafA_1$ or E-64d blocked GFP-LC3 degradation and caused a massive accumulation of GFP-LC3 dots (which represent autophagosomes) in mouse embryos [15]. Present results clearly showed that E-64 and rapamycin similarly increased autophagy activity during bovine oocytes maturation, it could be indirectly proved that inhibition of degradation of autophagosome is not occurred by E-64 treatment. Considering prolonged induction of autophagy can also induce apoptosis in bovine embryos [75]. These findings suggest that reduction in CTSB and induction of autophagy have a synergistic effect on improving embryo developmental competence by protecting blastocysts from preventing apoptosis, especially in good quality COCs-derived embryos, as the addition of E-64 + rapamycin decreased the apoptotic level compared to that in the rapamycin alone group. In the present experiment, I suggested that both autophagy induction and CTSB inhibition are potentially causes for an increase of developmental competence not only in poor quality oocytes, but also in good quality oocytes.

In the present study, the induction of autophagic activity was only administrated during oocyte maturation. Therefore, further study is necessary to investigate whether E-64 can also increase autophagic activity during different bovine embryonic developmental period.

Chapter 4 **Effect of regulation of autophagy and CTSB on developmental competence of bovine oocytes collected from transported ovaries**

Introduction

Recent decades, calf production has become an urgent issue due to a decrease in the number of cattle. Besides, along with the highly applied ET for improving fertility, a lack of embryo source has become increasingly prominent. In particular, bovine ovaries collected from slaughterhouses are accelerating the efficiency for IVF, and the entry of private companies are also increased resulted from competition for ovarian resources. Furthermore, it has become more common that ovaries collected from slaughterhouses in a remote place are transported over long distances to where IVF was performed. After slaughtering, the interruption of blood flow from the living body stops nutrients supply to ovarian tissue and eggs. Therefore, it is concerned that an accumulation of metabolites such as lactic acid produced by ovarian, and a decrease in oocyte viability results from deterioration of the follicular environment after slaughtering.

Based on the investigation of maturation and developmental potential of collected oocytes maintained from several to 24 h at 4-20 ℃, certain conditions are presented in the previous studies [89–91].

Moreover, it is unclear about the intracellular environment in the ovarian follicle during storage after slaughtered, which is considered involved in improving the oocyte viability.

Previous studies have identified that cytoplasmic proteolysis and low developmental potential were resulted from increased activity of lysosome-cysteine proteases, the CTSs, especially CTSB, in bovine oocytes and early embryos. It is also reported that the addition of E-64, a penetrating CTSB inhibitor, and vitamin B6 (pyridoxine) significantly improved embryonic development and quality [41,64,92]. Considering the CTSB activity associated with low-quality embryo, together with embryo quality reduction caused by intracellular proteolytic damage. On the other hand, induction of autophagy by its inducer rapamycin promoted preimplantation development of bovine embryos, increased porcine parthenotes development by decreasing apoptosis, as well as restored autophagy level and increased cloned mouse embryo viability [74–76].

In the present study, to improve IVC embryonic development, E-64, rapamycin or E-64 + rapamycin was added into IVM medium to reduce the damage of oocyte under long-term storage and transportation.

Materials and Methods

Storage and transportation of ovaries

Ovaries were collected in the afternoon at the Obihiro slaughterhouse, and arrived at Sapporo in the next afternoon. Ovaries were stored for 24 h under actual transport conditions at 20 °C. During transport process, constant-temperature transport container (Sampratec, iP-TEC) was used, in which a data logger was installed to record temperature every hour. In addition, the temperature change during transportation was also measured (Fig. 29).

Oocyte collection and IVM

After the transported ovaries arrived, the oocytes collection and IVM was described previously (Chapter 2), materials and methods section.

IVF and IVC

Described previously (Chapter 2), materials and methods section.

Nuclei staining and evaluation of total cell number of blastocysts

Blastocysts were fixed in 4% (w/v) PFA solution for 1 h. After fixation, blastocysts were washed three times in 0.2% PBS-PVA. Then, Hoechst 33342 (1 μg/ml, Sigma-Aldrich) was used to stain the nuclei, followed by mounting onto glass slides using mounting solution (Vectashield, Vector Laboratories). The fluorescence of the

hoechst-stained nuclei was detected using a fluorescence microscope (LAS X, Leica). The total cell number of blastocysts was calculated by total number of nuclei.

Experimental design

After collection, poor quality COCs were selected morphologically (as described in chapter 1) from the ovaries stored 24 h. Then CTSB inhibitor, E-64 and/or autophagy inducer, rapamycin, which greatly affects the recycling of intracellular proteins, were added into the maturation medium. Then, developmental competence of embryos was evaluated treated with rapamycin or E-64 alone or the mixture of them on day 2 and day 8, respectively.

Statistical analysis

Data are representative of at least three or five independent experiments. All data are shown as the mean ± standard error of the mean (SEM). Statistically significant differences were assessed by Student's *t-test* and one-way analysis of variance (ANOVA)-Tukey's Multiple Range Test implemented in Graphpad Prism® 7 Software (La Jolla, CA, USA). All percentage data were arcsine transformed prior to statistical analysis. *P* values < 0.001, < 0.01, or < 0.05 were considered statistically significant. *P* values < 0.1 were regarded as indicating a tendency.

Results

Comparison of developmental competence between freshly collected and transported poor quality COCs

The development competence between fresh low quality COCs (collected freshly after ovaried within 2 h) and low quality transported COCs was examined by cleavage rate on day 2 and blastocyst rate on day 8, respectively. As showed in Fig. 30, there was no significant difference between freshly collected and transported COCs.

Effects of autophagy induction together with CTSB inhibition on the developmental competence and quality of poor quality COCs collected from transported ovaries

IVM of COCs collected from ovaries transported 24h, followed by IVF and IVC, the cleavage rate and blastocyst rate were evaluated when treated with E-64 (1 μM) or rapamycin (100 nM) alone, or mixture with E-64 (1 μM) and rapamycin (100 nM). As a result, a higher tendency of blastocyst rate was found in day 8 treated with the mixture of E-46 and rapamycin than that treated with them separately. But the cleavage rate was not changed in the day 2 among each group (Fig. 31).

In addition, the combination of E-64 and rapamycin significantly increased total cell number of blastocysts than non-treatment group derived from COCs collected from transported ovaries. Whereas, the E-64 and rapamycin alone group did not increase total cell number compared to control group (Fig. 32). Consistent with this result, the

blastocysts derived from E-64 + rapamycin treatment group showed preferable morphology compared to control group (Fig. 33).

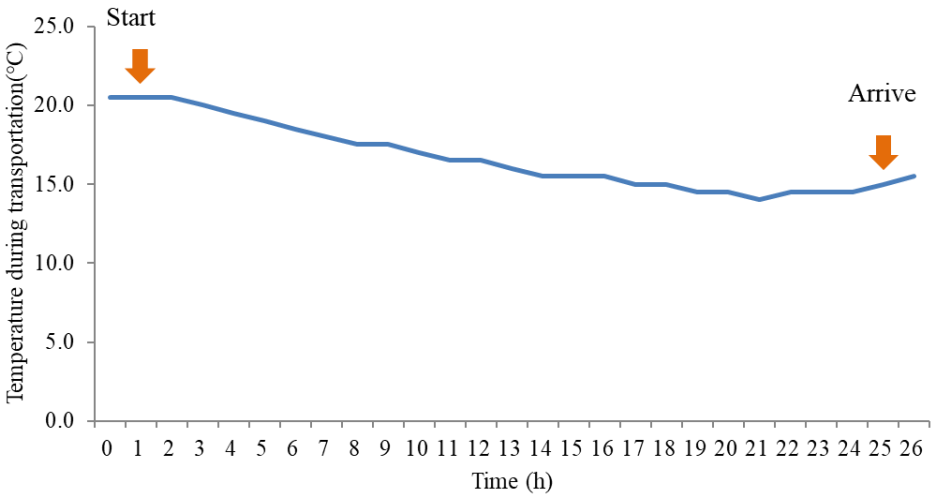

Fig. 29. Change of temperature during transportation.

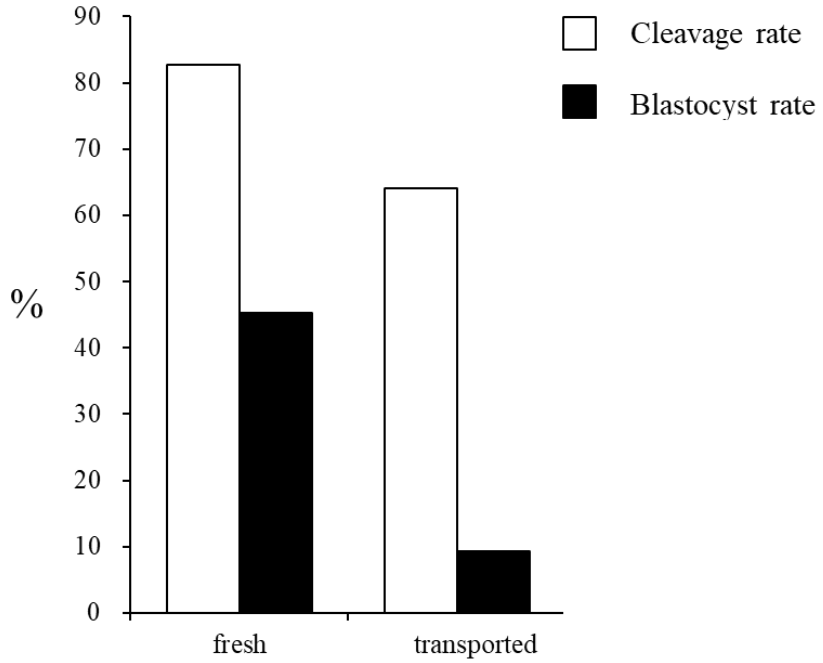

Fig. 30. Embryo developmental competence evaluated by cleavage rate and blastocyst rate derived from COCs collected from freshly collected and transported ovaries on day 2 and day 8, respectively. (fresh: ovaries obtained within 2 h). The experiment was repeated thrice.

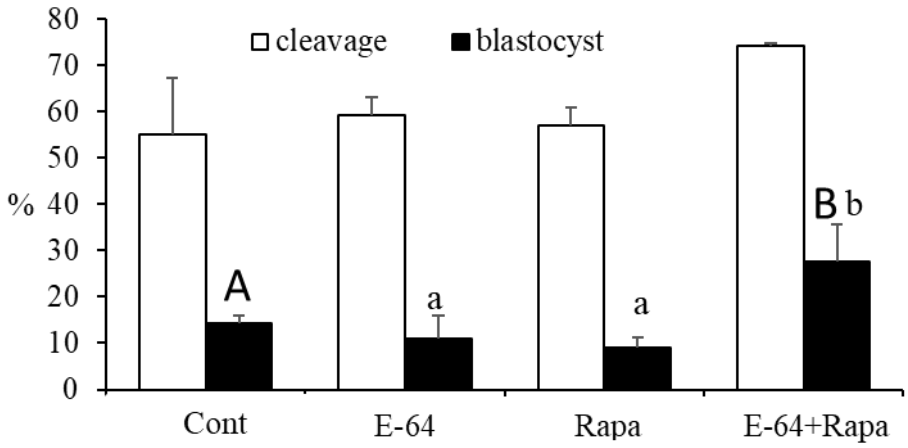

Fig. 31. Effect of E-64 (1 μM) and/or rapamycin (100 nM) treatment during IVM on subsequent cleavage and blastocyst rates of each indicated treatment group in poor quality COCs collected form transported ovaries. The experiment was repeated thrice. All data are shown as mean ± SEM. Bars with different letters indicate statistical difference (a vs. b：$P < 0.05$, A vs. B: $P = 0.1$). Cont: control; Rapa: rapamycin; E-64 + Rapa: E-64 + rapamycin.

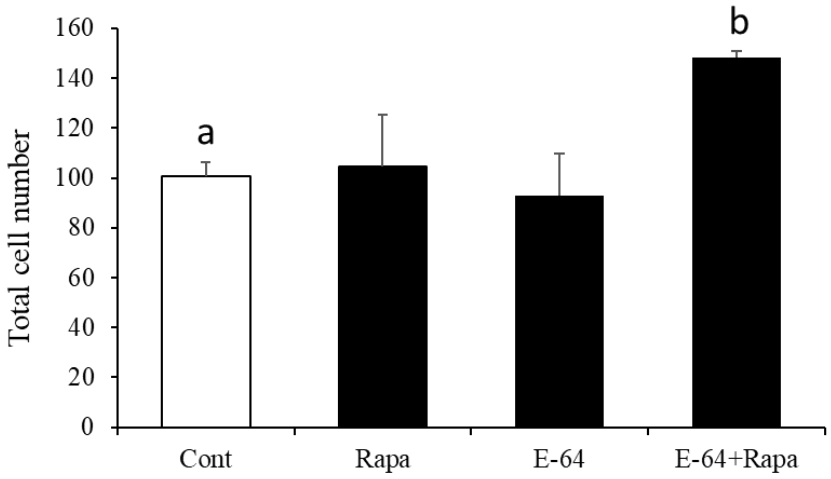

Fig. 32. Effect of E-64 (1 μM) and/or rapamycin (100 nM) treatment during IVM on subsequent embryo developmental quality derived from poor quality COCs collected form transported ovaries. Total cell number of day 8 blastocysts of each indicated treatment group (control group: Cont; E-64 group: E-64; rapamycin group: Rapa; E-64 + rapamycin group: E-64 + Rapa). The experiment was repeated three times. All data are shown as mean ± SEM. Bars with different letters indicate statistical difference ((a vs. b：$P < 0.01$).

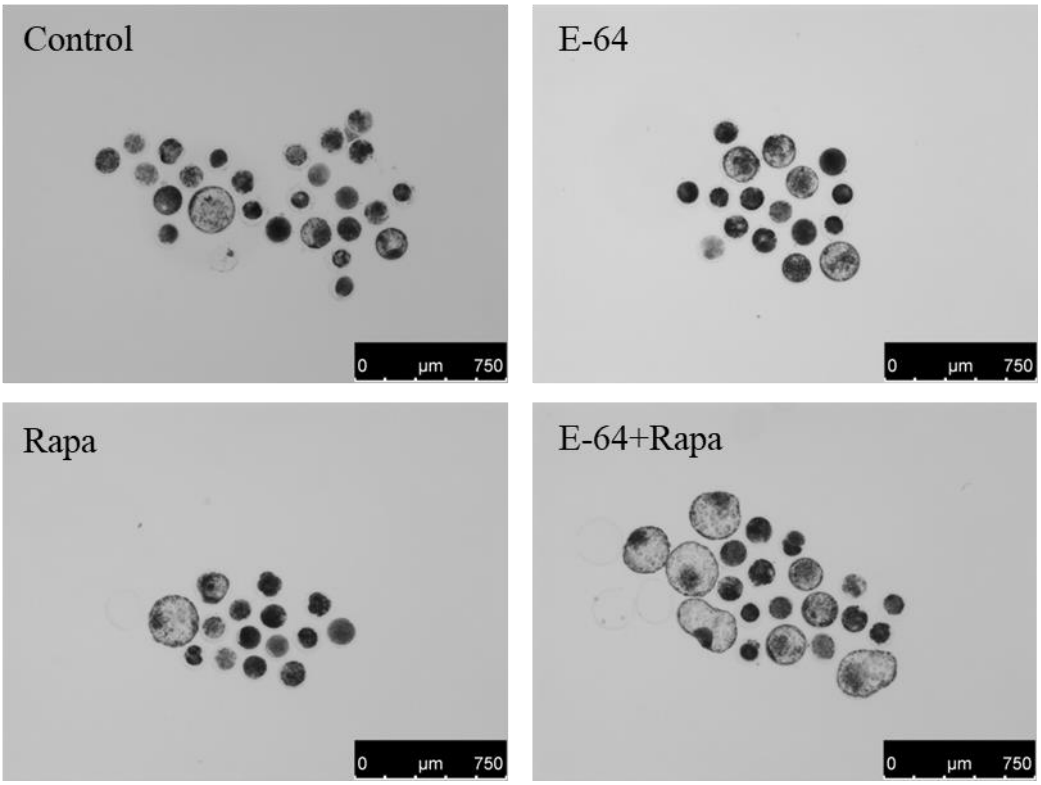

Fig. 33. Blastocysts derived from poor quality COCs collected from transported ovaries cultured with or without E-64 and/or rapamycin during IVM. Representative photographs of blastocysts in the indicated treatment groups (control group: Cont; E-64 group: E-64; rapamycin group: Rapa; E-64 + rapamycin group: E-64 + Rapa) derived from good quality COCs, respectively. The scale bar represents 750 µm.

Discussion

Many studies have been conducted on the preservation of bovine ovaries for efficient utilization of follicular oocytes. These results suggested that no effect on the creation of nuclear transfer embryos in the case of storage at 15-35°C for 3-4 h [91] and no effect on maturation rate, IVF rate and parthenogenesis after blastocysts after long-term storage at 10 °C for 24 h (25%, fresh: 27%) [93]. Moreover, blastocyst rate of 30 % and a cell count of 80-90 blastocysts were obtained when stored ovaries at both 20 °C and 35°C for 9 h [89]. One possibility of this discrepancy might be the parthenote activation to produce embryos and IVF. Also, all previous studies investigated the static storage of ovaries followed by oocyte collection. However, the present study included 24 h of transportation of ovaries. Therefore, vibration during the transportation might have caused the decline of developmental competence with decreasing the number of high quality COCs. At present, there was no previous report was found about the developmental competence of oocytes from long-time transported ovaries. Therefore, it is necessary to investigate the factors affecting the quality and developmental competence of oocytes from transported ovaries.

Rapamycin, autophagy-inducing reagent, is involved in the degradation of unnecessary proteins and micro-organs, and also associated with the reuse of amino acids during maturation culture of the oocytes collected from transported ovaries. Moreover, the mixed treatment of rapamycin and E-64 significantly increased the blastocyst rate compared to rapamycin or E-64 treatment alone during IVM. The

addition of rapamycin to the culture medium of bovine 1-cell stage embryos after IVF induces autophagy and a significant improvement in the blastocyst development rate. Furthermore, the number of autophagosomes involved in autophagic activation were regulated by CTSB [94]. Additionally, the developmental potential is improved by suppressing CTS activation due to low quality [64], freezing [95] or heat stress [63] which suggested a synergistic effect of CTS inhibition and autophagy regulation. However, the reason for no effect of the utilization of rapamycin and E-64 separately on embryonic developmental competence of bovine oocytes collected from transported ovaries needs further analysis.

Moreover, blastocyst rate could be rescued by CTS inhibitor and autophagy inducer via adjusting the culture environment and improving the overall survival of ovarian tissues, therefore, the mechanisms still need further examination.

Chapter 5 **General Discussion**

The blastocyst stage is an important stage for ET. During mammalian preimplantation embryonic development, after sperm penetrates into the oocyte, the embryo begins life as a totipotent, single cell organism. Subsequent cycle of the cell division and the formation of tight junctions between blastomeres results in a condition whereby the blastomeres on the outer face exhibits different patterns of cell polarity, gene expression and protein accumulation than blastomeres in the inner part of the embryo [96–99]. On the other hand, the non-polarized blastomeres in the inner part of the embryo are predestined to form the pluripotent ICM of the blastocyst. While, the polarized cells in the outer face of the embryo are destined to differentiate into the TE, which develops into extraembryonic membranes. Furthermore, the ICM contributes to form the fetus, whereas TE will contribute only to the placental tissues. ICM undergoes a second round of cell lineage specification to form the precursors of epiblast (EPI) and hypoblast (or primitive endoderm: PE) [100]. TE gives rise to parts of the placenta and the chorion. In bovine, PE develops to the primitive and secondary yolk sac [100] .

Previously, it has been revealed that CTSB inhibition by E-64 during IVM significantly increased TE cell number of blastocysts [41]. This is partly supported by CTSB activity and protein were significantly higher in ICM compared to TE in blastocysts. Consistent with the inhibition of CTSB resulted in increasing of TE cells

number, induction of autophagy by rapamycin also significantly increased TE ratio of bovine blastocysts [75]. Taken together, these results suggested that the lysosomal mediated autophagy machinery have a pivotal role in TE formation and further implantation in mammals. However, the present study did not show the inhibition of CTSB and induction of autophagy in ICM and TE cells number of blastocysts; and the synergetic effect of CTSB inhibition and autophagy induction was observed in good and poor quality COCs. Therefore, it is necessary to examine the ICM and TE cell number of blastocysts in the near future.

The present study indicated that autophagy activation was caused not only by rapamycin, but also caused by E-64 in poor rank of bovine oocytes during IVM. Although the autophagy activation by rapamycin/E-64 in good quality COCs was not detected, it is necessary to compare the autophagy activation mechanism in good and poor quality COCs. Moreover, the induction of autophagic activity was focused on oocyte maturation. It is necessary to investigate whether E-64, rapamycin, and E-64 + rapamycin can also increase autophagy during different bovine embryonic developmental process. In addition, the gene expression levels of *LC3α*, *LC3β*, *CTSB* and *caspase 3* were detected on COCs and oocytes after IVM , it is also necessary to investigate the effect of E-64, rapamycin, and E-64 + rapamycin on other autophagy related genes (such as *Atg 5*, *Atg 7* and *Beclin 1*) *CTSB* and *caspase 3, 7* during different embryonic developmental period.

Moreover, the effect of CTSB inhibition and autophagy induction of full-term

embryo development is not accomplished in present study. Furthermore, the ET should be performed in the future, especially the blastocysts derived from poor quality COCs.

Chapter 6 **Conclusion**

The overall results suggest that, embryo derived from poor quality COCs showed the lower developmental competence and quality by confirming the lower cleavage and blastocyst rate, and the total cell number, and higher TUNEL-positive cells of blastocyst compared to those of the good quality COCs derived embryos. In addition, higher autophagic activity was detected in good quality oocytes than poor grade oocytes, suggesting that autophagy is positively correlated with the oocyte quality. Furthermore, inhibition of autophagy resulted in decreasing developmental competence with the increase CTSB activity and *CTSB* and *caspase 3* gene expression. Taken together, these results indicated that developmental competence of embryo and quality is correlated with autophagy associated with CTSB activity and related gene expression in bovine oocytes.

Taking into account that CTSB inhibition increased embryo developmental competence and quality together with decreased apoptotic index of blastocysts by previous reports as well as autophagy inhibition decreased embryo development competence in chapter 2. Therefore, the effect of autophagy induction and CTSB inhibition on autophagic activity in poor quality oocyte after IVM was examined. Both E-64 (CTSB inhibitor) and rapamycin (autophagy inducer) increased autophagic activity of oocytes after IVM. In addition, E-64 treatment also increased the gene expression levels of *CTSB*. These results suggested that not only rapamycin, but also

CTSB mediated pathway can activate autophagy. Moreover, supplementation of 100 nM rapamycin alone significantly increased blastocyst rate supported by decreasing of TUNEL-positive cells number of blastocysts derived from poor quality COCs. However, these results were not observed in blastocysts derived from good quality COCs. Considering 10 nM rapamycin significantly increased bovine blastocyst rate and decreased the apoptosis of good quality embryos, it is concluded that the concentration of rapamycin is important on oocyte quality, that 10 nM is suitable for good quality COCs during IVM, whereas the higher concentration 100 nM is preferable for poor quality COCs. In contrast, as mentioned in chapter 3, combined administration of rapamycin and E-64 significantly increased blastocyst rate, total cell number and decreased TUNEL-positive cells derived both in good and poor quality COCs, especially for recuing poor quality COCs.

Finally, in chapter 4, combination treatment of rapamycin and E-64 was also applied in bovine oocytes collected from long-tine transported ovaries. The mixture of rapamycin and E-64 supplementation also increased the blastocyst rate with the increased number of total cells, suggesting that the combined effect of autophagy induction and CTSB inhibition can improve embryo development derived from long-transferred ovaries.

It is concluded that 1) autophagy can be one of the potential markers for evaluation of bovine oocyte quality, 2) embryonic developmental competence of embryo and quality is correlated with autophagy associated with CTSB activity and related gene expression

in bovine oocytes, 3) autophagy is not only activated by its inducer, rapamycin, but also by CTSB mediated pathway, 4) and the combined effect of autophagy induction and CTSB inhibition can contribute to improve quality and developmental competence of oocytes for ET in cattle.

References:

[1] Dominko T, First NL. Timing of meiotic progression in bovine oocytes and its effect on early embryo development. Mol Reprod Dev 1997;47:456–467.

[2] Rizos D, Ward F, Duffy P, Boland MP, Lonergan P. Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: Implications for blastocyst yield and blastocyst quality. Mol Reprod Dev 2002;61:234–248.

[3] Lindner GM, Wright RW. Bovine embryo morphology and evaluation. Theriogenology 1983;20:407–416.

[4] Scott L. The morphology of human pronuclear embryos is positively related to blastocyst development and implantation. Hum Reprod 2000;15:2394–2403.

[5] Tooze J, Hollinshead M, Ludwig T, Howell K, Hoflack B, Kern H. In exocrine pancreas, the basolateral endocytic pathway converges with the autophagic pathway immediately after the early endosome. J Cell Biol 1990;111:329–345.

[6] Liou W, Geuze HJ, Geelen MJ, Slot JW. The autophagic and endocytic pathways converge at the nascent autophagic vacuoles. J Cell Biol 1997;136:61–70.

[7] Berg TO, Fengsrud M, Strømhaug PE, Berg T, Seglen PO. Isolation and characterization of rat liver amphisomes: Evidence for fusion of autophagosomes with both early and late endosomes. J Biol Chem 1998;273:21883–21892.

[8] Nara A, Mizushima N, Yamamoto A, Kabeya Y, Ohsumi Y, Yoshimori T. SKD1

AAA ATPase-dependent endosomal transport is involved in autolysosome formation. Cell Struct Funct 2002;27:29–37.

[9] Mizushima N. Autophagy: Process and function. Genes Dev 2007;21:2861–2873.

[10] Mizushima N. The pleiotropic role of autophagy: From protein metabolism to bactericide. Cell Death Differ 2005;12:1535–1541.

[11] Mizushima N, Levine B. Autophagy in mammalian development and differentiation. Nat Cell Biol 2010;12:823–830.

[12] Kuma A, Hatano M, Matsui M, Yamamoto A, Nakaya H, Yoshimori T, Ohsumi Y, Tokuhisa T, Mizushima N. The role of autophagy during the early neonatal starvation period. Nature 2004;432:1032–1036.

[13] Komatsu M, Waguri S, Ueno T, Iwata J, Murata S, Tanida I, Ezaki J, Mizushima N, Ohsumi Y, Uchiyama Y, Kominami E, Tanaka K, Chiba T. Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. J Cell Biol 2005;169:425–434.

[14] Tsukamoto S, Kuma A, Murakami M, Kishi C, Yamamoto A, Mizushima N. Autophagy is essential for preimplantation development of mouse embryos. Science 2008;321:117–120.

[15] Tsukamoto S, Hara T, Yamamoto A, Kito S, Minami N, Kubota T, Sato K, Kokuno T. Fluorescence-based visualization of autophagic activity predicts mouse embryo viability. Sci Rep 2014;4:4533.

[16] Mohamed MM, Sloane BF. Multifunctional enzymes in cancer. Nat Rev Cancer

2006;6:764–775.

[17] Turk V, Stoka V, Vasiljeva O, Renko M, Sun T, Turk B, Turk D. Cysteine cathepsins: From structure, function and regulation to new frontiers. Biochim Biophys Acta 2012;1824:68–88.

[18] Stoka V, Turk B, Turk V. Lysosomal cysteine proteases: Structural features and their role in apoptosis. IUBMB Life 2005;57:347–353.

[19] Klose A, Zigrino P, Dennhöfer R, Mauch C, Hunzelmann N. Identification and discrimination of extracellularly active cathepsins B and L in high-invasive melanoma cells. Anal Biochem 2006;353:57–62.

[20] Turk B, Turk D, Turk V. Lysosomal cysteine proteases: More than scavengers. Biochim Biophys Acta 2000;1477:98–111.

[21] Olstein AD, Liener IE. Comparative studies of mouse liver cathepsin B and an analogous tumor thiol proteinase. J Biol Chem 1983;258:11049–11056.

[22] Oksjoki S. Differential expression patterns of cathepsins B, H, K, L and S in the mouse ovary. Mol Hum Reprod 2001;7:27–34.

[23] Bettegowda A, Patel OV., Lee KB, Park KE, Salem M, Yao J, Ireland JJ, Smith GW. Identification of novel bovine cumulus cell molecular markers predictive of oocyte competence: Functional and diagnostic implications. Biol Reprod 2008;79:301–309.

[24] Horvitz HR. Worms, life, and death (Nobel lecture). Chem Bio Chem 2003;4:697–711.

[25] Jacobson MD, Weil M, Raff MC. Programmed cell death in animal development. 1997;88:347–354.

[26] Danial NN, Korsmeyer SJ. Cell Death: Critical control points. Cell 2004;116:205–219.

[27] Jeong SY, Seol DW. The role of mitochondria in apoptosis. J Biochem Mol Biol 2008;41:11–22.

[28] Abrams JM. An emerging blueprint for apoptosis in Drosophila. Trends Cell Biol 1999;9:435–440.

[29] Vaux DL, Flavell RA. Apoptosis genes and autoimmunity. Curr Opin Immunol 2000;12:719–724.

[30] Wallace C, Unger S. A matter of life and death. Commun Int 1999;26:34.

[31] Yuan J, Yankner BA. Apoptosis in the nervous system. Nature 2000;407:802–809.

[32] Bursch W. The autophagosomal-lysosomal compartment in programmed cell death. Cell Death Differ 2001;8:569–581.

[33] Shi Y. Mechanisms of caspase activation and inhibition during apoptosis. Mol Cell 2002;9:459–470.

[34] Thornberry NA, Lazebnik Y. Caspases : Enemies within. Science 1998;281:1312–1317.

[35] Alnemri ES, Livingston DJ, Nicholson DW, Salvesen G, Thornberry NA, Wong WW, Yuan J. Human ICE/CED-3 protease nomenclature. Cell 1996;87:171.

[36] Riedl SJ, Shi Y. Molecular mechanisms of caspase regulation during apoptosis. Nat Rev Mol Cell Biol 2004;5:897–907.

[37] Aits S, Jäättelä M. Lysosomal cell death at a glance. J Cell Sci 2013;126:1905–1912.

[38] Tsukamoto S, Hara T, Yamamoto A, Ohta Y, Wada A, Ishida Y, Kito S, Nishikawa T, Minami N, Sato K, Kokubo T. Functional analysis of lysosomes during mouse preimplantation embryo development. J Reprod Dev 2013;59:33–39.

[39] Brackett BG, Bousquet D, Boice ML, Nonawick WJ, Evans JF, Dressel MA. Normal developmental following in vitro fertilization in the cow. Biol Reprod 1982;27:147–158.

[40] Krisher RL. The effect of oocyte quality on development. J Anim Sci 2004;82 E-Suppl:E14–23.

[41] Balboula AZ, Yamanaka K, Sakatani M, Hegab AO, Zaabel SM, Takahashi M. Cathepsin B activity is related to the quality of bovine cumulus oocyte complexes and its inhibition can improve their developmental competence. Mol Reprod Dev 2010;77:439–448.

[42] Sirard MA, Richard F, Blondin P, Robert C. Contribution of the oocyte to embryo quality. Theriogenology 2006;65:126–136.

[43] Santiquet NW, Develle Y, Laroche A, Robert C, Richard FJ. Regulation of gap-junctional communication between cumulus cells during in vitro maturation

in swine, a Gap-FRAP study. Biol Reprod 2012;87:1–8.

[44] Eppig JJ, Pendola FL, Wigglesworth K, Pendola JK. Mouse oocytes regulate metabolic cooperativity between granulosa cells and oocytes: Amino acid transport. Biol Reprod 2005;73:351–357.

[45] Fatehi AN, Roelen BAJ, Colenbrander B, Schoevers EJ, Gadella BM, Bevers MM, van den Hurk R. Presence of cumulus cells during in vitro fertilization protects the bovine oocyte against oxidative stress and improves first cleavage but does not affect further development. Zygote 2005;13:177–185.

[46] Su J, Wang Y, Li R, Peng H, Hua S, Li Q, Quan F, Guo Z, Zhang Y. Oocytes selected using BCB staining enhance nuclear reprogramming and the in vivo development of SCNT embryos in cattle. PLoS One 2012;7:1–10.

[47] Wongsrikeao P, Kaneshige Y, Ooki M, Taniguchi M, Agung B, Nii M, Otoi T. Effect of the removal of cumulus cells on the nuclear maturation, fertilization and development of porcine oocytes. Reprod Domest Anim 2005;40:166–170.

[48] de Matos DG, Furnus CC, Moses DF. Glutathione synthesis during in vitro maturation of bovine oocytes: Role of cumulus cells. Biol Reprod 1997;57:1420–1425.

[49] Gilchrist RB, Ritter LJ, Armstrong DT. Oocyte-somatic cell interactions during follicle development in mammals. Anim Reprod Sci 2004;82–83:431–446.

[50] Makabe S, Naguro T, Stallone T. Oocyte-follicle cell interactions during ovarian follicle development, as seen by high resolution scanning and transmission

electron microscopy in humans. Microsc Res Tech 2006;69:436–449.

[51] Tatemoto H, Sakurai N, Muto N. Protection of porcine oocytes against apoptotic cell death caused by oxidative stress during in vitro maturation: Role of cumulus cells. Biol Reprod 2000;63:805–810.

[52] Glick D, Barth S, Macleod KF. Autophagy: Cellular and molecular mechanisms. J Pathol 2010;221:3–12.

[53] Mizushima N, Komatsu M. Autophagy: Renovation of cells and tissues. Cell 2011;147:728–741.

[54] Barth S, Glick D, Macleod KF. Autophagy: Assays and artifacts. J Pathol 2010;221:117–124.

[55] Mohamed MM, Sloane BF. Cysteine cathepsins: Multifunctional enzymes in cancer. Nat Rev Cancer 2006;6:764–775.

[56] Vancompernolle K, Van Herreweghe F, Pynaert G, Van De Craen M, De Vos K, Totty N, Sterling A, Flers W, Vandernabeele P, Grooten J. Atractyloside-induced release of cathepsin B, a protease with caspase-processing activity. FEBS Lett 1998;438:150–158.

[57] Thornberry NA. The caspase family of cysteine proteases. Br Med Bull 1997;53:478–490.

[58] Turk B, Stoka V. Protease signalling in cell death: caspases versus cysteine cathepsins. FEBS Lett 2007;581:2761–2767.

[59] Brill A, Torchinsky A, Carp H, Toder V. The role of apoptosis in normal and

abnormal embryonic development. J Assist Reprod Genet 1999;16:512–519.

[60] Brison DR, Schultz RM. Apoptosis during mouse blastocyst formation: Evidence for a role for survival factors including transforming growth factor α. Biol Reprod 1997;56:1088–1096.

[61] Bossy-Wetzel E, Newmeyer DD, Green DR. Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization. EMBO J 1998;17:37–49.

[62] Kim SH, Zhao MH, Liang S, Cui XS, Kim NH. Inhibition of cathepsin B activity reduces apoptosis by preventing cytochrome c release from mitochondria in porcine parthenotes. J Reprod Dev 2015;61:261–268.

[63] Balboula AZ, Yamanaka K, Sakatani M, Kawahara M, Hegab AO, Zaabel SM, Takahashi M. Cathepsin B activity has a crucial role in the developmental competence of bovine cumulus-oocyte complexes exposed to heat shock during in vitro maturation. Reproduction 2013;146:407–417.

[64] Balboula AZ, Yamanaka K, Sakatani M, Hegab AO, Zaabel SM, Takahashi M. Intracellular cathepsin B activity is inversely correlated with the quality and developmental competence of bovine preimplantation embryos. Mol Reprod Dev 2010;77:1031–1039.

[65] Hazeleger NL, Hill DJ, Stubbing RB, Walton JS. Relatiohship of morphology and follicular fluid environment of bovine oocytes to their developmental

potential in vitro. Theriogenology 1995;43:509–522.

[66] Lee JS, Lee GM. Monitoring of autophagy in Chinese hamster ovary cells using flow cytometry. Methods 2012;56:375–382.

[67] Mizushima N, Ohsumi Y, Yoshimori T. Autophagosome formation in mammalian cells. Cell Struct Funct 2002;27:421–429.

[68] Liu J, Xia H, Kim M, Xu L, Li Y, Zhang L, Cai Y, Norberg HV, Zhang T, Furuya, T, Jin M, Zhu Z, Wang H, Yu J, Li Y, Hao Y, Choi A, Ke H, Ma D, Yuan J. Beclin1 controls the levels of p53 by regulating the deubiquitination activity of USP10 and USP13. Cell 2011;147:223–234.

[69] Rubio Pomar FJ, Teerds KJ, Kidson A, Colenbrander B, Tharasanit T, Aguilar B, Roelen BA. Differences in the incidence of apoptosis between in vivo and in vitro produced blastocysts of farm animal species: A comparative study. Theriogenology 2005;63:2254–2268.

[70] Shen XH, Jin YX, Liang S, Kwon JW, Zhu JW, Lei L, Kim NH. Autophagy is required for proper meiosis of porcine oocytes maturing in vitro. Sci Rep 2018;8:1–12.

[71] Kang R, Zeh HJ, Lotze MT, Tang D. The Beclin 1 network regulates autophagy and apoptosis. Cell Death Differ 2011;18:571–580.

[72] Scarlatti F, Maffei R, Beau I, Codogno P, Ghidoni R. Role of non-canonical Beclin 1-independent autophagy in cell death induced by resveratrol in human breast cancer cells. Cell Death Differ 2008;15:1318–1329.

[73] Wada Y, Sun-Wada GH, Kawamura N, Aoyama M. Role of autophagy in embryogenesis. Curr Opin Genet Dev 2014;27:60–66.

[74] Xu YN, Shen XH, Lee SE, Kwon JS, Kim DJ, Heo YT, Cui XS, Kim NH. Autophagy influences maternal mRNA degradation and apoptosis in porcine parthenotes developing in vitro. J Reprod Dev 2012;58:576–584.

[75] Song BS, Yoon SB, Kim JS, Sim BW, Kim YH, Cha JJ, Choi SA, Min HK, Huh JW, Lee SR, Kim SH, Koo DB, Choo YK, Kim HM, Kim SU. Induction of autophagy promotes preattachment development of bovine embryos by reducing endoplasmic reticulum stress. Biol Reprod 2012;8:1–11.

[76] Shen X, Zhang N, Wang Z, Bai G, Zheng Z, Gu Y, Wu YS, Liu H, Zhou DJ, Lei L. Induction of autophagy improves embryo viability in cloned mouse embryos. Sci Rep 2015;5:17829.

[77] Sriraman V, Richards JS. Cathepsin L gene expression and promoter activation in rodent granulosa cells. Endocrinology 2004;145:582–591.

[78] Song ZH, Yu HY, Wang P, Mao GK, Liu WX, Li MN, Wang HN, Shang YL, Liu C, Xu ZL, Sun QL, Li W. Germ cell-specific Atg7 knockout results in primary ovarian insufficiency in female mice. Cell Death Dis 2015;6:e1589.

[79] Zhou J, Peng X, Mei S. Autophagy in ovarian follicular development and atresia. Int J Biol Sci 2019;15:726–737.

[80] Lee J, Park JI, Yun JI, Lee Y, Yong H, Lee ST, Park CK, Hyun SH, Lee GS, Lee E. Rapamycin treatment during in vitro maturation of oocytes improves

embryonic development after parthenogenesis and somatic cell nuclear transfer in pigs. J Vet Sci 2015;16:373–380.

[81] Li M, Khambu B, Zhang H, Kang JH, Chen X, Chen D, Vollmer L, Liu PQ, Vogt A, Yin XM. Suppression of lysosome function induces autophagy via a feedback down-regulation of MTOR complex 1 (MTORC1) activity. J Biol Chem 2013;288:35769–35780.

[82] Djavaheri-Mergny M, Maiuri MC, Kroemer G. Cross talk between apoptosis and autophagy by caspase-mediated cleavage of Beclin 1. Oncogene 2010;29:1717–1719.

[83] Cirman T, Orešić K, Mazovec GD, Turk V, Reed JC, Myers RM, Salvesen GS, Turk B. Selective disruption of lysosomes in HeLa cells triggers apoptosis mediated by cleavage of Bid by multiple papain-like lysosomal cathepsins. J Biol Chem 2004;279:3578–3587.

[84] Stoka V, Turk B, Schendel SL, Kim TH, Cirman T, Snipas SJ, Ellerby LM, Bredesen D, Freeze H, Abrahamson M, Bromme D, Krajewski S, Reed JC, Yin XM, Turk V, Salvesen GS. Lysosomal protease pathways to apoptosis: Cleavage of Bid, not pro-caspases, is the most likely route. J Biol Chem 2001;276:3149–3157.

[85] Lamparska-Przybysz M, Gajkoswa B, Motyl T. Cathepsins and BID are involved in the molecular swithch between apoptosis and autophagy in breast cancer MCF-7 cells exposed to campothecin. J Physiol Pharmacol 2005:Suppl3:159–

179.

[86] Fouladi-Nashta AA, Alberio R, Kafi M, Nicholas B, Campbell KHS, Webb R. Differential staining combined with TUNEL labelling to detect apoptosis in preimplantation bovine embryos. Reprod Biomed Online 2005;10:497–502.

[87] Hosseini SM, Hajian M, Asgari V, Forozanfar M, Abedi P, Nasr-Esfahani MH. Novel approach of differential staining to detect necrotic cells in preimplantation embryos. Iran J Fertil Steril 2007;1:103–106.

[88] Eisenberg-Lerner A, Bialik S, Simon HU, Kimchi A. Life and death partners: Apoptosis, autophagy and the cross-talk between them. Cell Death Differ 2009;16:966–975.

[89] Iwata H, Hayashi T, Sato H, Kimura K, Kuwayama T, Monji Y. Modification of ovary stock solution with magnesium and raffinose improves the developmental competence of oocytes after long preservation. Zygote 2005;13:303–308.

[90] Iwata H, Inoue J, Kimura K, Kuge T, Kuwayama T, Monji Y. Comparison between the characteristics of follicular fluid and the developmental competence of bovine oocytes. Anim Reprod Sci 2006;91:215–223.

[91] Wang YS, Zhao X, Su JM, An ZX, Xiong XR, Wang LJ, Liu J, Quan FS, Hua S, Zhang Y. Lowering storage temperature during ovary transport is beneficial to the developmental competence of bovine oocytes used for somatic cell nuclear transfer. Anim Reprod Sci 2011;124:48–54.

[92] Aboelenain M, Balboula AZ, Kawahara M, El-Monem Montaser A, Zaabel SM,

Kim SW, Nagano M, Takahashi M. Pyridoxine supplementation during oocyte maturation improves the development and quality of bovine preimplantation embryos. Theriogenology 2017;91:127–133.

[93] Matsushita S, Tani T, Kato Y, Tsunoda Y. Effect of low-temperature bovine ovary storage on the maturation rate and developmental potential of follicular oocytes after in vitro fertilization, parthenogenetic activation, or somatic cell nucleus transfer. Anim Reprod Sci 2004;84:293–301.

[94] Man SM, Kanneganti TD. Regulation of lysosomal dynamics and autophagy by CTSB/cathepsin B. Autophagy 2016;12:2504–2505.

[95] Pezhman M, Hosseini SM, Ostadhosseini S, Rouhollahi Varnosfaderani S, Sefid F, Nasr-Esfahani MH. Cathepsin B inhibitor improves developmental competency and cryo-tolerance of in vitro ovine embryos. BMC Dev Biol 2017;17:1–9.

[96] Eckert JJ, Fleming TP. Tight junction biogenesis during early development. Biochim Biophys Acta 2008;1778:717–728.

[97] Marikawa Y, Alarcon VB. Establishment of trophectoderm and inner cell mass lineages in the mouse embryo. Mol Reprod Dev 2009;76:1019–1032.

[98] Zernicka-Goetz M, Morris SA, Bruce AW. Making a firm decision: Multifaceted regulation of cell fate in the early mouse embryo. Nat Rev Genet 2009;10:467–677.

[99] Rossant J, Tam PPL. Blastocyst lineage formation, early embryonic asymmetries

and axis patterning in the mouse. Development 2009;136:701–713.

[100] Kuijk EW, van Tol LTA, van de Velde H, Wubbolts R, Welling M, Geijsen N, Roelen BA. The roles of FGF and MAP kinase signaling in the segregation of the epiblast and hypoblast cell lineages in bovine and human embryos. Development 2012;139:871–882.

Acknowledgments

First and foremost, I would like to show my deepest gratitude to my supervisor, professor & Dr. Masashi Takahashi for giving me a chance to study in Hokkaido University. I wish to express my gratitude for his grate guidance and valuable encouragement throughout my study, without his enlightening instruction, impressive kindness and patience this degree would not have been possible.

A word of special heartfelt thanks is given to Associate Prof. Manabu Kawahara for support and encouragement of my study in Sapporo. I would like to express deepest gratitude for Dr. Hanako Bai, for support and encouragement of my study in Sapporo. I am very grateful to appreciate Dr. Mansour ABOELENAIN and Dr. Ahmed Zaky BALBOULA for their valuable suggestions and comments, and valuable encouragement in my study. I express my great thank to Dr. Takashi Fujii and Dr. Satoru Moriyasu for sharing their valuable experimental materials. I am very grateful to thanks to Prof. Yasuo Kobayashi and Associate Prof. Satoshi Koike in laboratory of Animal Function and Nutrition for giving me opportunity to sharing the machineries. I want to acknowledge all the members of Laboratory of Animal Genetics and Reproduction for their kindness and friendships.

At last but not least my thanks would go to my beloved family in China and my wife in Sapporo for their continuous encouragement and great spiritual support.

Jianye LI

Supplementary List

Table 1. List of primers used for qRT-PCR

Genes	GenBank accession number	Primer Sequence (5'-3')	Product length (bp)
CTSB	NM_174031.2	CACTTGGAAGGCTGGACACA GCATCGAAGCTTTCAGGCAG	141
Caspase3	NM_001166521.1	CCCGTGGAACACCTGATCGCCAA CCCGATGCCGATCTCCCCGTA	277
LC3α	NM_001046175.1	TGTCAACATGAGCGAGTTGGT AGGAAGCCATCCTCGTCCTT	156
LC3β	NM_001001169.1	CGAGAGCAGCATCCTACCAA TGAGCTGTAAGCGCCTTCTT	151
H2AFZ	NM_174809.2	AGAGCCGGTTTGCAGTTCCCG TACTCCAGGATGGCTGCGCTGT	116

10×PBS	**(g/500ml)**
NaCl (Wako)	40 g
KCl (Wako)	1 g
Na_2HPO_4・$12H_2O$ (Wako)	14.405 g
KH_2PO_4 (Wako)	1 g

Autoclaved, store at Room Temperature

×1000 $CaCl_2$・$2H_2O$	**(g/10ml)**
$CaCl_2$・$2H_2O$ (Kanto Chemical, Tokyo, Japan)	1.057g

Store at 4˚C

×1000 $MgCl_2 \cdot 6H_2O$	(g/10ml)
$MgCl_2 \cdot 6H_2O$ (Kanto Chemical)	3.303g

Store at 4˚C

Antibiotic	1.5 ml
Penicillin (Nacalai tesque)	150000 U
Streptomycin (Nacalai tesque)	0.15 g

Penicillin:1533 U/mg ≈ 0.09784g

Divided into 0.5μl tube, 50μl/tube, store at -20˚C

FSH Stock	1 ml
FSH (Kyoritsu Medicine Manufacture Pharmacy, Tokyo, Japan)	20 AU
1x PBS	1 ml

Divided into 0.5μl tube, 10μl/tube, store at -20 ˚C, protect from light

PB1	100 ml
10×PBS	10 ml
Glucose (Wako)	0.1 g
Sodium Pyruvate (Wako)	0.0036 g
×1000 $CaCl_2 \cdot 2H_2O$	40.0μl
×1000 $MgCl_2 \cdot 6H_2O$	94.6μl
Antibiotic	50 μl
BSA (Sigma-Aldrich)	0.4 g

Sterile filtered, Store at 4˚C

TCM199	**(g/10 ml)**
199 (Gibco)	0.098 g
HEPES (Sigma-Aldrich)	0.0298 g
$NaHCO_3$ (Wako)	0.022 g
Cysteamine (Sigma-Aldrich)	1 µl
Antibiotic	5 µl
FSH Stock	10 µl
FCS (PAA The Cell Culture Company, Pasching, Austria)	500 µl

Sterile filtered before using, store at 4˚C

B.O. Stock	**(g/100 ml)**
NaCl	3.27265 g
KCl	0.14985 g
$NaH_2O_4 \cdot 2H_2O$	0.05725 g
$MgCl_2 \cdot 6H_2O$	0.05285 g
$CaCl_2 \cdot 2H_2O$	0.16515 g
Glucose	1.25905 g
Phenol red (Sigma-Aldrich)	50 µl

Store at 4˚C

B.O. + teophyllin working solution	**(g/100ml)**
$NaHCO_3$	0.3108 g
Na-pyruvate	0.0138 g
Theophyllin	0.045 g
Stock	10 ml
Antibiotic	50 µl

Sterile filtered, store at 4 ˚C

Bovine IVD101	**10 ml**
IVD101 (Research Institute for the Functional Peptides)	9.5 ml
FBS	500 µl
Antibiotic	5 µl

Sterile filtered, divided, store at 4 ˚C

Insulin Stock	**1 ml**
Bovine Insulin (Sigma-Aldrich)	0.01 g
Acetic Acid (Kanto Chemical)	10 µl
Autoclaved Water	1 ml

Store at 4˚C

10% PVA	**50 ml**
PVA (Sigma-Aldrich)	5 g

Autoclaved, store at 4˚C

40% PFA	**1 ml**
Paraformaldehyde (Wako)	0.4 g
1x PBS	1 ml

Store at 4˚C for 1 week

0.5% Proteinase	**(g/5ml)**
Proteinase (Wako)	0.025 g
PB1	5 ml

Centrifugation at 1800rpm for 5 min, remove the supernatant, divided 50ul/tube, store at -20˚C

PBS + 0.2%PVA	**50 ml**
10%PVA	100 µl
1x PBS	50 ml

Store at 4˚C

1% TritonX-100	**5 ml**
TritonX-100 (Wako)	50 µl
PBS+PVA	5 ml

Store at 4˚C for 1 week

Washing solution	**50 ml**
TritonX-100 (Wako)	50 µl
BSA (Sigma-Aldrich, A9418)	150 mg
1x PBS	50 ml

Store at 4˚C

0.1% Tween 20 in PBS	**10 ml**
Tween 20 (Wako)	10 µl
1x PBS	10 ml

Store at 4˚C