—Original Article—

Effect of autophagy induction and cathepsin B inhibition on developmental competence of poor quality bovine oocytes

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Abstract. The present study investigated the effect of autophagy induction and cathepsin B (CTSB) inhibition on developmental competence of poor quality oocytes. Bovine cumulus oocyte complexes (COCs) were classified as good or poor according to their morphology. Autophagy activity was detected in good and poor germinal vesicle (GV) oocytes. Then E-64, a CTSB inhibitor, rapamycin (Rapa), an autophagy inducer, and combined administration was achieved during *in vitro* maturation (IVM) of poor quality COCs followed by detection of autophagy activity. In the next experiment, E-64, Rapa, and E64 + Rapa, were added during IVM to good and poor quality COCs followed by *in vitro* fertilization and culture for 8 days to investigate whether inhibition of CTSB and/or induction of autophagy improve embryonic development and quality. Autophagy activity was significantly lower in poor quality GV oocytes than in good quality ones. E-64, Rapa and E-64 + Rapa treatment during IVM significantly increased autophagy activity in poor quality oocytes. Addition of Rapa in good quality COCs did not increase the blastocyst rate, whereas E-64 increased the blastocyst rate and total cell number (TCN) with decreasing TUNEL-positive cells. In contrast, Rapa treatment in poor quality COCs significantly increased the blastocyst rate and TCN with decreasing TUNEL-positive cells. These results indicate oocyte quality has different responses to intracellular autophagy induction and CTSB activity control by potential autophagy and catabolic status, however, synergetic effect of autophagy induction and CTSB inhibition can increase developmental competence of both good and poor quality COCs, especially rescue effect in poor quality COCs.
Key words: Autophagy, Cathepsin B, Cow, Developmental competence, Oocyte quality

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Many strategies have been employed to improve the efficiency of *in vitro* production (IVP) of cattle embryos [1]. However, the overall success remains lower than that of *in vivo* ones. Although many factors influence the efficiency and quality of the IVP technology, oocyte quality is a major factor. Oocyte quality determines early embryonic survival, developmental competence, establishment and maintenance of pregnancy, fetal development, and adult health [2]. Therefore, it is necessary to modulate the *in vitro* maturation system to rescue poor quality oocytes with subsequent improvement in the efficiency and quality of embryos produced *in vitro*.

Autophagy is an intracellular bulk degradation process in which

a portion of the cytoplasm is sequestered in autophagosomes and subsequently degraded upon fusion with lysosomes [3–5]. Autophagy plays a critical role during fertilization and is essential for embryonic differentiation and development in mice. Embryos derived from *Atg5* (autophagy-related gene 5) knocked-out oocyte fertilized by *Atg5*-null sperm failed to develop beyond the 4- and 8-cell stage but could develop if they were fertilized by wild-type sperm [6]. In addition, 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 [7–9]. Moreover, autophagic activity has been demonstrated as an indicator for selecting good quality mouse embryos by imaging fluorescence intensity of GFP-LC3, an autophagy marker and autophagic activity declined in poor quality embryos, probably because of a decline in the activity of lysosomal hydrolases [10].

Cathepsin B (CTSB), a lysosomal cysteine protease, plays a pivotal role in the degradation of intracellular proteins in lysosomes [11], and in regulating various biological processes such as cell death,

proliferation, migration, and cancer [12]. CTSB is a key player in the initiation of the apoptotic pathway through activating initiator caspases rather than executioner caspases, either directly or indirectly [13]. Specifically, CTSB activates caspases indirectly by inducing mitochondrial membrane degradation, resulting in translocation of apoptosis-inducing components from the mitochondria to the cytoplasm [14]. Indeed, inhibition of CTSB activity decreased apoptosis by preventing cytochrome c release from mitochondria in porcine parthenotes [15]. CTSB is detected in different types of cells, including the bovine cumulus oocyte complex (COCs) and embryos [15–17]. Recent studies revealed higher CTSB expression and activity in poor quality bovine and porcine embryos. Importantly, inhibition of CTSB activity increased the developmental competence of both bovine and porcine preimplantation embryos [15, 18]. 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 *in vitro* fertilization (IVF) [18, 19]. This indicates that lysosomal CTSB regulation is a promising strategy to improve the quality of embryos produced *in vitro*.

Therefore, the inverse relationship between CTSB and autophagy led us to investigate the effect of CTSB inhibition and autophagy induction during *in vitro* maturation (IVM) on the developmental competence of bovine embryos, especially for those derived from poor quality oocytes.

Materials and Methods

Oocyte collection and IVM

Bovine ovaries were collected from a local abattoir and transported to the laboratory at 20°C. The ovaries were washed several times in sterile saline. COCs were aspirated from follicles (2–8 mm in diameter) using a disposable 18-gauge needle attached to a 10-ml syringe and washed in PB1 medium. The COCs were matured in TCM-199 [(Gibco, Grand Island, NY, USA) supplemented with 10 μM cysteamine (Sigma Aldrich, St. Louis, MO, USA), 10% (v/v) fetal bovine serum (FBS) (PAA Laboratories, QLD, Australia), 0.5 mg/ml follicle-stimulating hormone (Kyoritsu Seiyaku, Tokyo, Japan), 100 U/ml penicillin (Nacalai Tesque, Kyoto, Japan), and 100 U/ml streptomycin (Nacalai Tesque)] covered with liquid paraffin (Nacalai Tesque) for 22 h in a humidified atmosphere containing 5% CO_2 in air.

IVF and in vitro culture

Frozen semen was thawed in warm water (38.5°C) for 30 sec. Spermatozoa were washed by centrifugation at 600 *g* for 7 min in Brackett and Oliphant medium containing 2.5 mM theophylline (Wako Pure Chemical Industries, Osaka, Japan) 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 make a final concentration of 5×10^6/ml of spermatozoa pellet. The matured COCs were transferred to IVF100 sperm medium and cultured for 18 h at 38.5°C in a humidified atmosphere of 5% CO_2 and 5% O_2 in air. After fertilization, presumptive zygotes were denuded by pipetting to remove cumulus cells and cultured in Bovine IVD101 medium

(Research Institute for the Functional Peptides) containing 5% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin at 38.5°C in a humidified atmosphere of 5% CO_2 and 5% O_2 in air for 8 days. The cleavage and blastocyst rates were recorded on day 2 and 8, respectively.

Detection of autophagy activity in good and poor quality germinal vesicle (GV) oocytes and E-64, rapamycin, E-64 + rapamycin-treated poor quality metaphase II (MII) oocytes

Autophagy activity in good and poor quality GV oocytes as well as E-64 (Research Institute for the Functional Peptides), rapamycin (Enzo Life Sciences, Farmingdale, NY, USA), 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, GV and MII oocytes were incubated 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 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 phosphate buffered saline containing 0.2% polyvinyl alcohol (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, Germany).

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 blastocysts. Blastocysts were fixed in 4% (w/v) paraformaldehyde solution for 1 h. After fixation, blastocysts were washed thrice in PBS containing 0.2% polyvinylalcohol (PVA), and then permeabilized in 0.2% PBS-PVA containing 0.5% Triton X-100 for 20 min. Embryos were washed thrice 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 thrice with 0.2% PBS-PVA for 5 min each, followed by mounting onto glass slides using mounting solution (Vectashield, Vector Laboratories, Burlingame, CA, USA). The fluorescence of the fragmented DNA was detected using a fluorescence microscope (LAS X, Leica, Germany) 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, a subset of COCs was denuded by repeated pipetting, and at least five of GV-denuded oocytes were sampled of each replication to assess autophagy activity. The remaining COCs were divided into two groups according to their morphological quality as described previously [18, 21]. COCs were evaluated based on the cumulus layer and the status of their cytoplasm. Briefly, COCs with compact and complete cumulus layer and evenly granulated and homogenous cytoplasm were classified as good quality oocytes. In

contrast, COCs showing abnormal cumulus distribution and ooplasmic features were considered as poor quality oocytes (Supplementary Fig. 1: online only). 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 culture, cleavage and blastocyst rates were assessed on day 2 and 8, respectively. Total cell number and apoptotic index were evaluated on day 8 embryos.

A subset of *in vitro* matured COCs were denuded by repeated pipetting in the presence of 0.1% hyaluronidase for 2–3 min. At least of five of 1 μM E-64, 100 nM rapamycin and 1 μM E-64 + 100 nM rapamycin-treated MII-denuded oocytes was sampled and evaluated for autophagy activity of each replication.

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

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

Autophagy activity was evaluated in good and poor quality GV oocytes using Cyto-ID dye. The fluorescence intensity of Cyto-ID reflects the autophagy flux. Autophagy activity was clearly observed in the cytoplasm of all oocytes. A stronger fluorescence of autophagy activity was observed in good GV oocytes than in poor quality oocytes (Fig. 1A). The intensity of autophagic fluorescence was significantly lower ($P < 0.001$) in poor quality oocytes than in good quality ones (Fig. 1B)

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

Considering that autophagy activity is lower in poor quality oocytes (Fig. 1), we sought to elucidate whether E-64, rapamycin as well as 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 autophagy activity. Autophagy activity was clearly observed in the cytoplasm of all oocytes (Fig. 2A). 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 autophagy activity when compared to that in the control (Fig. 2B), indicating that E-64 and rapamycin can induce autophagy activity in bovine oocyte system.
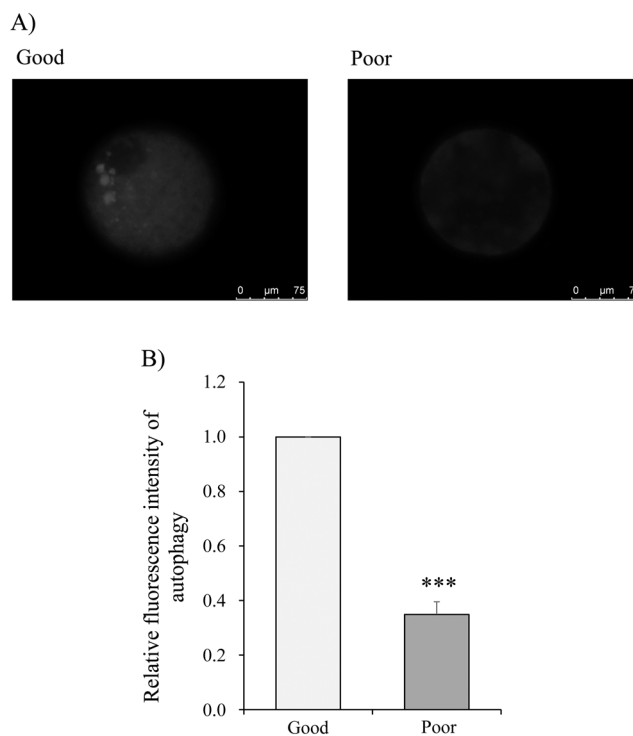

Fig. 1. Autophagy activity in bovine (germinal vesicle) GV stage oocytes of good and poor quality. Autophagy activity was barely detectable in the poor quality oocytes, but strong in the good quality oocytes (A). Relative fluorescence intensity of autophagy (representing autophagy activity) between good and poor quality oocytes (B). The experiment was repeated thrice. The scale bar represents 75 μm. All data are shown as mean ± SEM. Asterisks indicate statistical difference (*** $P < 0.001$) between good and poor quality oocytes.

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 [18]. Supplementation of E-64 and/or rapamycin during IVM improved the morphological quality of day 8 blastocysts in each indicated group (Supplementary Fig. 2: online only). Addition of rapamycin alone and E-64 + rapamycin treatment groups improved embryonic development especially derived from poor quality COCs. Even though there was no significant difference in cleavage 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 a high increasing tendency compared to that in the control group (31.5 ± 2.4) derived from good quality COCs (Table 1). In the case of 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
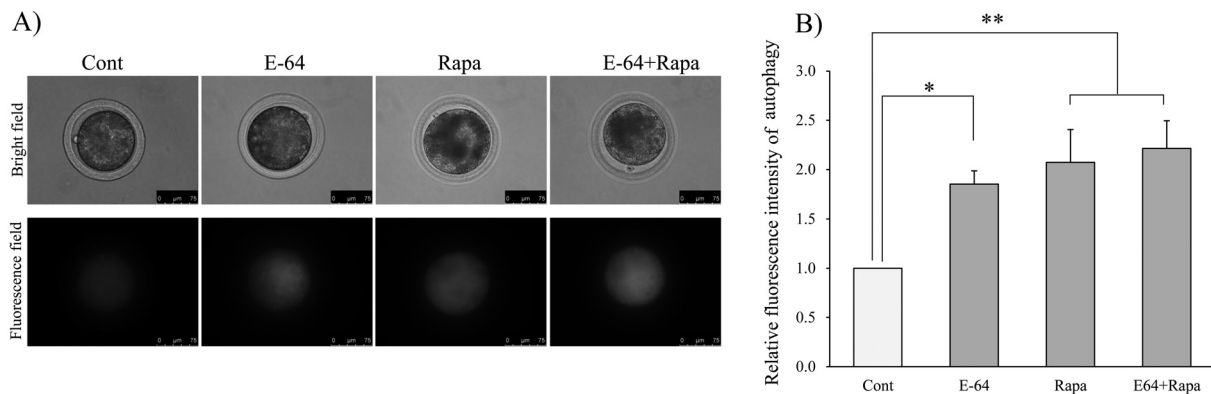
A)

B)

Fig. 2. Effect of E-64, rapamycin and E-64 + rapamycin treatment on autophagy activity in *in vitro* matured poor quality MII oocytes. Representative images of autophagy activity (A) of bright field images and corresponding fluorescent images of poor quality MII oocytes after *in vitro* maturation of the indicated treatment groups (Cont, control group; E-64, E-64 group; Rapa, rapamycin group; E-64 + Rapa, E-64 + rapamycin group). Relative fluorescence intensity of autophagy (representing autophagy activity) between the indicated treatment groups (B). The experiment was repeated thrice. The scale bar represents 75 μm. All data are shown as mean ± SEM. Asterisks indicate statistical difference (* $P < 0.05$, ** $P < 0.01$).

Table 1. 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 cumulus oocyte complexes (COCs) derived bovine embryos

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

Data are represented as the mean ± SEM of five replicates. a *vs.* b: $P < 0.05$, a *vs.* c: $P = 0.09$. Rapa, rapamycin; E-64 + Rapa, E-64 + rapamycin.

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

Treatment	No. of replication	No. of inseminated oocytes	Cleavage rate (%)	Blastocyst rate (%)
Control	5	119	46.7 ± 7.6 [a]	10.8 ± 2.4 [A]
E-64	5	107	67.0 ± 4.6	19.2 ± 4.4
Rapa	5	111	73.4 ± 4.6 [b]	29.4 ± 5.2 [B]
E-64 + Rapa	5	112	69.3 ± 4.7 [b]	30.2 ± 6.3 [C]

Data are represented as the mean ± SEM of five replicates. a *vs.* b: $P < 0.05$, A *vs.* B: $P = 0.06$, A *vs.* C: $P < 0.05$. Rapa, rapamycin; E-64 + Rapa, E-64 + rapamycin.

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.). 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 1 and 2).

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

To investigate the quality of blastocysts produced from good and poor quality COCs, total cell number and TUNEL apoptotic assay were evaluated (Fig. 3A and 3B). E-64 alone and E-64 + rapamycin treatment promoted the embryo quality derived from good quality COCs, whereas both E-64 or rapamycin alone and E-64 + rapamycin treatment improved the embryo quality derived from poor quality COCs (Supplementary Fig. 2). 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. 3C). 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. 3E). In addition, E-64 + rapamycin treatment significantly decreased the apoptotic index compared to that in the rapamycin alone group (P < 0.05) (Fig. 3E). 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. 3D), 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. 3F). In addition, the total cell number of blastocysts derived from no treatment COCs (control group) was significantly (P = 0.01) higher in good quality blastocysts than in poor quality ones, which is supported by the apoptotic index that showed a high tendency (P = 0.07) in poor quality blastocysts compared to that in the good quality group (Supplementary Fig. 3: online only).

Discussion

Multiple events, especially oocyte quality, affect the ability of the putative fertilized zygote to develop to the blastocyst stage, to implant, 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 recognized 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 mechanism by which follicular atresia is induced in mammalians [24]. However, it has been demonstrated that autophagy altered follicular development, for example, the ovaries of *Atg7* deficient mice contain fewer germ cells and primordial follicles [25]. Moreover, autophagy led to programmed cell death in which regulation of follicular atresia was induced by oocytes [26]. We therefore propose the hypothesis that inhibition of CTSB and/or induction of autophagy alters the embryonic developmental competence, especially for rescuing the poor quality oocytes in bovines.

As reported in previous studies, higher CTSB activity was observed in poor quality bovine and porcine GV oocytes and inhibition of its activity by E-64 (1 µM) decreased CTSB activity and improved embryonic development competence [15, 18]. However, autophagy activity in good and poor quality GV oocytes in bovines remains unclear. Therefore, to test our hypothesis, we first examined autophagy activity in the GV stage of good and poor quality oocytes and rapamycin-induced effect of autophagy activity in poor quality oocytes during IVM. Higher autophagy activity was observed in good quality oocytes and rapamycin increased autophagy activity in poor quality MII oocytes during IVM. The higher autophagy detected in good quality GV stage oocytes is consistent with the findings of Tuskamoto *et al*. who observed a higher GFP-LC3 fluorescence in poor quality mouse embryos [10]. Because total GFP-LC3 fluorescence decreases when autophagy is active [27], embryos with low or high GFP-LC3 fluorescence can be classified as good or poor, respectively. Moreover, investigations showed the higher autophagy activity (good

quality) embryos showed the prominent 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 [10]. 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 [6], which is different with our results. One possibility of this discrepancy might be the different species. On the other hand, the MII oocytes were collected *in vivo* during ovulation in mouse; however, in present study, the GV stage oocytes were collected from the small follicles and *in vitro* matured MII oocytes were used. It is necessary to compare the quality of oocytes that were selectively ovulated and non-selectively collected followed by IVM. Our research showed that the induction of autophagy activity after rapamycin treatment in poor quality MII oocytes is also partly supported by the abundance of mRNA transcripts of *Beclin* 1 and *LC3* in poor quality porcine MII oocytes after rapamycin treatment during IVM [28]. Interestingly, our results clearly showed that E-64 treatment during IVM also increased autophagy activity. The present 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 [29]. Taken together, these findings suggest that CTSB and autophagy activity is negatively and positively correlated with the oocyte quality, respectively, and can be used as an indicator for oocyte quality. Moreover, our results indicated that the autophagy activation is caused not by rapamycin, but also caused by E-64 in poor grade of bovine oocytes during *in vitro* maturation. Although we did not detect the autophagy activation by rapamycin/E64 in good quality COCs in the present study, 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 [30, 31]. 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 autophagy activity on oocyte quality. Subsequently, we 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 and 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
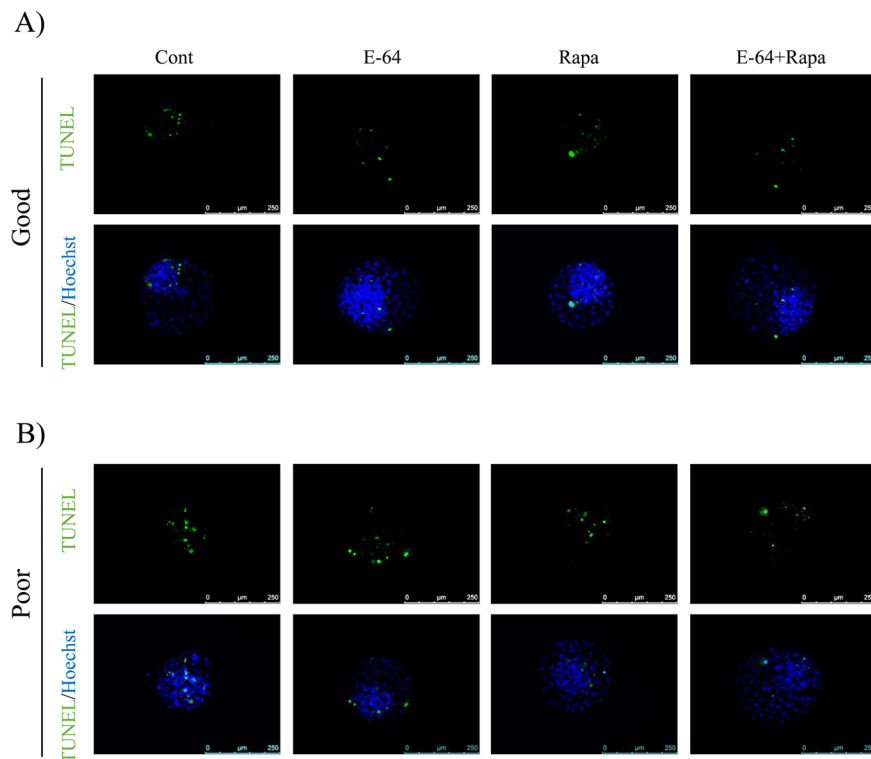
A)

B)

Fig. 3. Improvement in blastocyst quality through inhibition of CTSB activity and induction of autophagy during IVM of IVP-produced good and poor quality embryos. Representative images of apoptosis detection analysis using good (A) and poor (B) quality blastocysts of the indicated treatment groups (Cont, control group; E-64, E-64 group; Rapa, rapamycin group; E-64 + Rapa, E-64 + rapamycin group). 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). Total cell number of each indicated group from good (C) and poor (D) quality blastocysts. Apoptotic index of each indicated group from good (E) and poor (F) quality blastocysts. The experiment was repeated five times. n: No. of blastocyst examined. The scale bar represents 250 μm. All data are shown as mean ± SEM. Asterisks indicate statistical difference (* P < 0.05, ** P < 0.01, *** P < 0.001).

cells of blastocysts after IVM and *in vitro* culture (IVC) [18]. In addition, treatment of bovine or 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 [15, 17]. 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 increase compared to that in the control group derived from good quality COCs. However, interestingly, in contrast to the good quality group, rapamycin treatment significantly increased the cleavage rate and showed the 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.

Our results were consistent with those of previous studies showing that rapamycin (10 nM) treatment of early stage embryos enhanced autophagy levels of *in vitro* produced bovine, porcine, and somatic cell nuclear transfer produced mouse embryos and increased their subsequent embryo viability [7–9], and also decreased the apoptosis levels of blastocysts [7, 8]. 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 explained by 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 [8, 28]. Thus, taking into account that higher autophagy 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 [32].

Finally, we also analyzed the synergistic 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. We 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 or show a strong tendency for a higher blastocyst rate
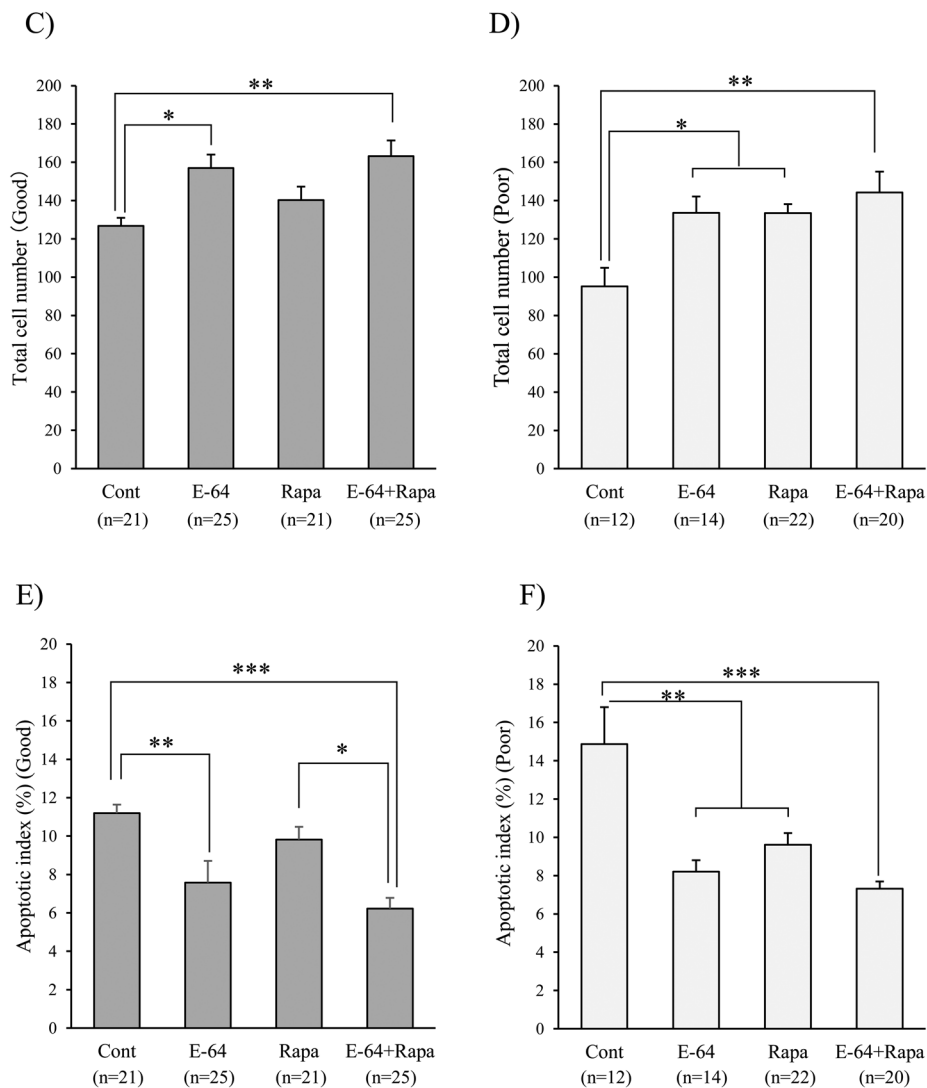
C)

D)

E)

F)

Fig. 3. continued.

compared to that in the control group derived from poor and 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. 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 [10]. Our results clearly showed that E-64 and rapamycin similarly increased autophagy activity during bovine oocyte 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 [8]. 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 study, the induction of autophagy activity was focused on oocyte maturation. However, it is necessary to investigate whether E-64 can also increase autophagy activity during different bovine embryonic developmental period .

The molecular mechanisms by which CTSB and autophagy affect apoptosis in the mammalian embryo are not yet clear. CTSB can induce the apoptotic pathway through activating initiator caspases rather than executioner caspases, either directly or indirectly [13]. CTSB was found to active caspases indirectly through induction of mitochondrial membrane degradation, which led to the release of cytochrome-c, a pro-apoptogenic component, and apoptosis inducing factors [14]. The translocation of cytochrome-c from mitochondria is

a crucial step in apoptosis pathways [33–35]. Furthermore, autophagy is generally thought of as a survival mechanism; however, recent research has shown that the autophagy-specific protein ATG5 can be cleaved following death stimuli, and the cleavage product can translocate from the cytosol to the mitochondria to trigger cytochrome-c release and caspase activation, resulting in mitochondria-mediated apoptosis [36]. These insights led us to conclude that the regulation of suppression of cytochrome-c release induced apoptosis in good quality IVP embryos is strongly associated with cooperation between CTSB activity and prolonged autophagy activity.

In contrast, cystatin c is generally considered an important inhibitor of cysteine protease activity because of its potent inhibitory effect on CTSB [37]. Recent research revealed induction of autophagy by cystatin c plays a protective role in murine primary cortical neurons and neuronal cell lines under conditions of neuronal challenge by inducing autophagy via the inhibition of mammalian target of rapamycin (mTOR) [38] and mTOR forms the catalytic core of at least two functionally distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [8]. In the present study, we induced autophagy using rapamycin, a special prophylactic for mTOR that inhibits mTOR activity [39] thereby inducing autophagy [40]. Indeed, rapamycin preferentially inhibits mTORC1 by interfering with complex assembly and thereby suppresses mTORC1 downstream targets, including ribosomal S6 protein kinase 1 (S6K1), eukaryotic initiation factor 4E-binding protein 1 (4EBP1), and UNC-51-like kinases (ULKs). In mTORC1-mediated signaling pathway, S6K1 and 4EBP1 are involved in cell proliferation [41], while autophagy is associated with the activation of ULKs [42]. Therefore, rapamycin treatment can influence cell proliferation as well as autophagy. On the other hand, it has been demonstrated that BafA$_1$ and E64d were able to inhibit mTORC1 signaling system thereby active autophagy [29]. Taken together, in the present study, addition of E-64 and rapamycin significantly increased embryo development competence accompanied by the reduced apoptotic levels which might be caused by the inhibition of CTSB-mediated autophagy inducing machinery via inhibition of the mTORC1 signaling pathway, especially in poor quality embryos. In the present study, dose dependent combination effect of E-64 and rapamycin was not fully accomplished. Therefore, it is still necessary to clarify the suitable combined concentration of each reagent for the effect of developmental competence of COCs.

In conclusion, our results suggest that autophagy can be a useful marker of oocyte quality and inhibition of CTSB and induction of autophagy by E-64 and rapamycin, respectively, during IVM improves developmental competence and quality, especially of poor quality oocytes.

Acknowledgments

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