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Cathepsin B Inhibitor, E-64, Affects Preimplantation Development, Apoptosis and Oxidative Stress in Pig Embryos

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ABSTRACT

Cathepsin B is abundantly expressed peptidase of the papain family in the lysosomes, and closely related to the cell degradation system such as apoptosis, necrosis and autophagy. Abnormal degradation of organelles often occurs due to release of cathepsin B into the cytoplasm. Many studies have been reported that relationship between cathepsin B and intracellular mechanisms in various cell types, but porcine embryos has not yet been reported. Therefore, this study evaluated the effect of cathepsin B inhibitor (E-64) on preimplantation developmental competence and quality of porcine embryos focusing on apoptosis and oxidative stress. The expression of cathepsin B mRNA in porcine embryos was gradually decreased in inverse proportion to E-64 concentration by using real-time RT-PCR. When putative zygotes were cultured with E-64 for 24 h, the rates of early cleavage and blastocyst development were decreased by increasing E-64 concentration. However, the rate of blastocyst development in 5 μM treated group was similar to the control. On the other hand, both the index of apoptotic and reactive oxygen species (ROS) of blastocysts were significantly decreased in the 5 μM E-64 treated group compared with control. We also examined the mRNA expression levels of apoptosis related genes in the blastocysts derived from 5 μM E-64 treated and non-treated groups. Expression of the pro-apoptotic *Bax* gene was shown to be decreased in the E-64 treated blastocyst group, whereas expression of the anti-apoptotic *Bcl-xL* gene was increased. Taken together, these results suggest that proper inhibition of cathepsin B at early development stage embryos improves the quality of blastocysts, which may be related to not only the apoptosis reduction but also the oxidative stress reduction in porcine embryos.

(Key words : E-64, Apoptosis, ROS, Porcine, Blastocyst)

INTRODUCTION

Pigs are a precious resource not only livestock but also experimental animal in the biotechnology. Also, it has a physiological and immunological mechanism similar to that of humans (Cooper, 2012). Therefore, development of genetically modified pig is used as human disease models and bioreactors. It was already been used extensively to study a wide variety of life science (Nagashima *et al*., 2007). However, the production of *in vitro* produced (IVP) porcine embryos has not been fully achieved. The poor quality of porcine embryos has been obstacle to the generation of offspring. Accordingly, improvement of culture medium is indispensable to produce good quality porcine embryos *in vitro*. Thus, many researchers have been performed with the aim of improving the quality of IVP porcine embryos as using new technique and adding protein sources (Gajda *et al*., 2008, Taka *et al*., 2005).

Various factors such as osmolarity, oxygen tension and enzymes affect preimplantation developmental competence of porcine embryos (Karja *et al*., 2004). Among the many great deals, lysosomal cysteine proteases are abundant expressed peptidase of the papain family in intracellular (Frlan and Gobec, 2006). It plays an important role in the degradation of intracellular proteins and in the oogenesis of vertebrates (Carnevali *et al*., 2006). In particular, cathepsin B is induced the apoptotic pathway through activating initiator caspases as a caspase zymogens activator (Balboula *et al*., 2010a). It is also implicated in a various disease involving tissue-remodeling states such as inflammation, cancer invasion, arthritis and osteoporosis (Martin *et al*., 2010). Although

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the mode of the action of cathepsin B is not fully understood, it has a strong relationship with apoptosis in many cell types (Bhoopathi *et al*., 2010).

Apoptosis is process of programmed cell death in multicellular organisms, which plays a crucial role during embryonic development. The programmed cell death is essential for appropriate embryonic development and degradation of unnecessary cells, it is a biological mechanism related to cytochrome *c* liberation or stimulation of death receptors by the activation of cysteine protease family, leading to typical morphologic changes termed apoptosis (Zuzarte *et al*., 2007). Furthermore, cysteine proteases-triggered apoptosis is mediated via apoptotic signaling mechanisms such as anti-thymocyte globulin induced apoptosis, which is associated with the release of cathepsin B from the lysosomes into the cytoplasm (Michallet *et al*., 2003). Especially, abnormal degradation of organelles often occurs due to excessive release of cathepsin B, and the inappropriate apoptosis induced the serious diseases and disorders (Vancompernolle *et al*., 1998).

Living organisms has energy generating mechanisms in suit with a surrounding environment. In aerobic organisms the energy is produced from the electron transfer system in the mitochondria, then reactive oxygen species (ROS) are commonly generated by activity of cellular metabolism (Indo *et al*., 2007). In addition, previous study reported that cysteine proteases stimulate ROS generation in dermal dendritic cells and epithelial cells (Tang *et al*., 2010). The generated ROS are linked to the apoptotic process such as chromatin condensation, DNA fragmentation, cytoplasmic membrane blebbing and cell shrinkage (Robertson and Orrenius, 2002). Especially, the generation of intracellular ROS made a big impact on mitochondrial apoptosis by the disruption of redox homeostasis (Park *et al*., 2012).

In embryogenesis, hyper-apoptosis is one cause of increasing poor quality embryos, and excessive cathepsin B activity is related to induce apoptosis. Furthermore, suitable regulation of cathepsin B was greatly improves the preimplantation developmental competence of embryos and increases the number of good quality embryos in bovine (Balboula *et al*., 2010b). Thus, the regulation of cathepsin B by various inhibitors may be closely related to the quality of porcine embryos. The E-64 is cathepsin B inhibitor of epoxide, which can irreversibly inhibit lysosomal cysteine proteases (Barrett *et al*., 1982). Many experimental data suggested about that correlation of between cathepsin B inhibition and intracellular mechanism in various cell types (Ni *et al*., 2012, Reich *et al*., 2009), but the effect of E-64 has not yet been reported in porcine embryos. Therefore, in this study examined the effect of cathepsin B inhibitor (E-64) in preimplantation development competence and qualities of porcine embryos focusing on apoptosis and oxidative stress.

MATERIALS AND METHODS

Chemicals

Unless noted otherwise, all chemicals used in the present study were purchased from Sigma Aldrich Korea (Yongin, Korea).

In Vitro Maturation (IVM) and *In Vitro* Fertilization (IVF)

Porcine ovaries were obtained from at a local slaughterhouse and transported to the laboratory in 0.9% saline supplement with 75 mg/ml potassium penicillin G at approximately 30~35℃. Immature cumulus-oocyte complexes (COCs) were aspirated from follicles between 3 and 6 mm in diameter using an 18-gauge needle into a disposable 10 ml syringe (Funahashi *et al*., 1994). Undamaged COCs with the same quality cytoplasm and surrounded by cumulus cells were selected by mouth pipetting and then washed three times in TL-HEPES medium. Overall, approximately 50-60 COCs were matured in 500 μl of IVM medium in a 4-well multidish (Nunc, Roskilde, Denmark) at 38.5℃ and under 5% CO_2 in air. BSA free North Carolina State University (NCSU) 23 medium supplemented with 10% follicular fluid, 0.57 mM cysteine, 10 ng/ml β-mercaptoethanol, 10 ng/ml epidermal growth factor (EGF), 10 IU/ml pregnant mare serum gonadotropin (PMSG) and 10 IU/ml human chorionic gonadotropin (hCG) was used for oocyte maturation (Petters and Wells, 1993). After culturing for 22 h, COCs were washed three times and then further cultured in PMSG and hCG-free maturation medium for 22 h. After completion of IVM, IVF of porcine oocytes was performed as described by Abeydeera and Day (1997). The IVF medium, modified Tris-buffered medium (mTBM), consisted of 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2$, 5 mM sodium pyruvate, 11 mM glucose, 20 mM Tris, 2.5 mM caffeine sodium benzoate and 1 mg/ml BSA. Fresh semen was kindly supplied once a week by an artificial insemination company (Darby Porcine AI Center, Anseong, Korea) and kept at 17℃ for 5 days. Semen was then washed three times by centrifugation with Dulbecco's phosphate buffered saline (DBPS, Gibco BRL, Grand Island, NY) supplemented with 1 mg/ml BSA (Fraction V), 100 mg/ml penicillin G, and 75 mg/ml streptomycin sulfate. At the end of washing, the spermatozoa were resuspended in mTBM at pH 7.8. Oocytes were washed three times in mTBM with 2.5 mM caffeine sodium benzoate and 1

mg/ml BSA (fatty acid free), after which they were placed into 48 μl of mTBM under paraffin oil. Next, 2 μl of diluted spermatozoa were added to a 48 μl drop of medium containing 15~20 oocytes to give a final concentration of 1.5×10^5 sperms/ml. Finally, the oocytes were co-incubated with spermatozoa for 6 h at 38.5℃ and under 5% CO_2 in air.

In Vitro Culture (IVC)

For all experiments, the embryos were cultured in 50 μl drops of PZM-3 medium with 3 mg/ml BSA at 38.5 ℃ and under 5% CO_2 in air. At this stage, cathepsin B inhibitor (E-64; 0, 5, 10 and 20 μM) was added to the IVC medium. After 24 h of culture, cleaved embryos were further cultured in 50 μl drops of PZM-3 medium without E-64 at same conditions for 5 days. Blastocyst formation was evaluated under a stereomicroscope (Olympus) at 6 days after insemination.

TUNEL Assay

The number of apoptotic nuclei in the blastocysts was detected using an In Situ Cell Death Detection Kit, Fluoresce (Roche Diagnostics GmbH, Mannheim, Germany). Blastocysts were recovered from IVC after 6 days, washed three times with 0.1% PVA-PBS and then fixed in 4% (v/v) paraformaldehyde/PBS solution for 1 h at 4℃. For membrane permeabilization, the fixed embryos were incubated in PBS containing 0.1% (v/v) Triton X-100 for 30 min at 4℃, after which they were incubated in TUNEL reaction medium for 1 h at 38.5℃ in the dark and then washed and transferred to 2 mg/ml of DAPI and mounted on glass slides. Whole-mount embryos were examined under an epifluorescence microscope (Olympus) using the TUNEL assay and DAPI and the numbers of apoptotic nuclei and total number of nuclei were determined.

Measurement of ROS

The level of ROS in each embryo was measured using the difluorodihydrofluorescein diacetate method (H_2-DCFDA; Invitrogen, Molecular Probes, Willow, USA) described previously (Hashimoto *et al*., 2000). At day 6, IVP blastocysts were recovered and used for the experiment. After three washes in IVC medium, blastocysts were transferred into IVC medium containing 5 mM H_2DCFDA for 20 min at 38.5℃. A stock solution of H_2DCFDA dissolved in dimethylsulfoxide (DMSO) was then diluted in IVC medium, after which the permeabilized blastocysts in H_2DCFDA were washed three times with 0.1% PVA-PBS and placed into a 50 μl drop covered with mineral oil. The recorded fluorescent images were then detected by epifluorescence microscopy (Olympus). The number of pixels was measured using Image J software version 1.38 (National Institutes of Health, Bethesda, MD). Background intensities were measured and subtracted from final values. A total of 30 blastocysts were examined in each treatment group.

Gene Expression Analysis by Real-Time RT-PCR

The mRNA was isolated from 20 blastocysts on Day 6 of IVC using a Dynabeads mRNA direct kit (DYNAL; Invitrogen, Carlsbad, CA, USA) and RNeasy plus micro kit (Qiagen, Chatsworth, CA, USA) according to the manufacture's protocol. The mRNA was reversed transcribed in a 20 ml reaction mixture containing 8 ml RNA, 50 ng/ml random hexamers, 10 mM dNTP mix, 10X RT buffer, 25 mM $MgCl_2$, 0.1 M DTT, RNase OUT 40 U/μl, and superscript III RT 200 U/μl. The reaction was then carried out by subjecting the samples to the following conditions: 65℃ for 5 min, 50℃ for 50 min, 85℃ for 5 min and then cooling to 4℃ until use as a template for PCR. Real-time RT-PCR was performed using an Applied Stratagene mx 3,000p QPCR System (San Diego, CA, USA) in a final reaction volume of 20 μl with SYBR Green (Applied Biosystems, Foster City,

Table 1. Primer sequences for real-time RT-PCR

Gene (Gene bank accession number)	Primer sequence	Annealing temperature (℃)	Expected PCR size (bps)
CTSB (NM001097458)	F: GACATGCTCACCTGTTGTGG R: CCATCAAGTCTCCTGTGACG	62	418
Bax (AJ606301)	F: AAGCGCATTGGAGATGAACT R: CGATCTCGAAGGAAGTCCAG	60	251
Bcl-xL (AF213205)	F: AGGGCATTCAGTGACCTGAC R: TGGATCCAAGGCTCTAGGTG	60	242
GAPDH (U07786)	F: GGGCATGAACCATGAGAAGT R: AAGCAGGGATGATGTTCTGG	60	230

CA, USA). For each quantification, a 2 μl aliquot of the reverse transcribed reaction was used. All samples were quantified simultaneously during the same run using the housekeeping gene *GAPDH*. The primers used for real-time RT-PCR are shown in Table 1. The amplification program was as follows: pre-incubation for fast-start polymerase activation at 95℃ for 10 min, followed by 45 cycles of denaturation at 95℃ for 40 s, annealing at 58~60℃ for 40 s, and elongation at 72℃ for 45 s, followed by measurement of fluorescence. After the end of the last cycle, a melting curve was generated by initiating fluorescence acquisition at 72℃ and taking measurements at every 0.1℃ until 95℃ was reached.

Statistical Analysis

All experiments were repeated at least three times. All percentage data obtained in the present study are presented as the mean±standard deviation (SD). Preimplantation development, the cell number of blastocysts and mRNA expression in the experiments were analyzed by Student's *t*-tests and Duncan's multiple range test using the GLM prosedure in the SAS. A probability of $p<0.05$ was considered significant.

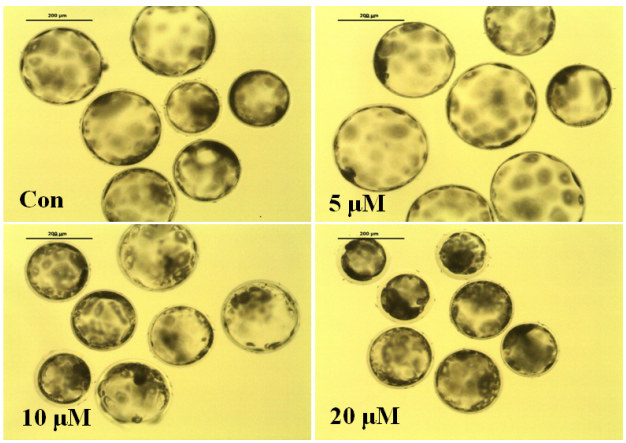
RESULTS

Effect of E-64 on the Developmental Competence and Cathepsin B Genes (CTSB) Expression of Porcine Blastocysts

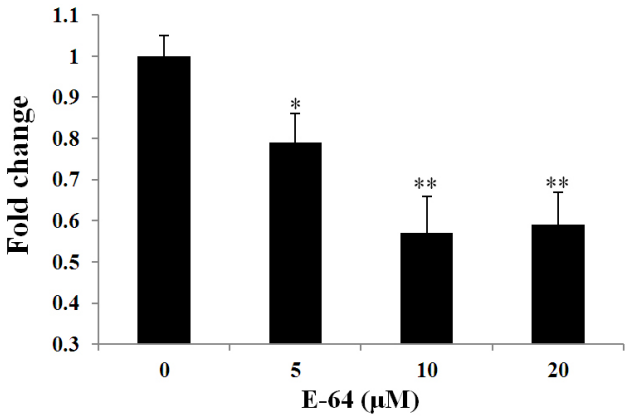
To investigate the relationship between inhibition of cathepsin B at early cleavage stages and preimplantation developmental competence, the E-64 is treated for 24 h in IVC medium. As shown in Table 2, the production rates of blastocyst in 10 and 20 μM E-64 treated groups were negatively affected when compared with control group ($p<0.05$), whereas the developmental rate of blastocyst in 5 μM E-64 treated group was similar to the control. Otherwise, no difference was observed in cleavage rates among the E-64 treatment and non-treatment groups. We also investigated that whether E-64 can inhibit the *CTSB* mRNA expression in porcine embryos. Blastocysts were derived from the E-64 treated and non-treated groups for 24 h in IVC medium (Fig. 1A). The expressions of *CTSB* mRNA in the E-64 treated groups were lower compared with control group (Fig. 1B, $p<0.05$).

Apoptotic Patterns in Porcine Blastocysts Derived from E-64 Treated and Non-Treated Groups

As shown in Fig 2A and B, the total cell numbers in



(A)



(B)

Fig. 1. Representative photographs image (A) and relative mRNA abundance of *CTSB* gene (B) in porcine blastocysts. Data are the mean±SD. Statistically significant differences are indicated by asterisks ($p<0.05$). Scale bars=200 μm.

10 and 20 μM E-64 treated groups were less than that of control group ($p<0.05$), but the total cell numbers in 5 μM E-64 treated group were similar to the control group. Furthermore, the apoptotic index in the 5 μM E-64 treated group was showed the lowest level than other groups ($p<0.05$). The expression levels of apoptosis-related genes, *Bax* and *Bcl-xL*, were analyzed in blastocysts derived from the 5 μM E-64 treated groups using real-time RT-PCR (Fig. 2C). The expression of *Bax* was significantly lower in blastocysts derived from 5 μM E-64 treatment group than in the control ($p<0.05$). Otherwise, the expression level of *Bcl-xL* was significantly higher in blastocysts derived from 5 μM E-64 treatment group than in the control ($p<0.05$).

Effects of E-64 Treatment on Expression Levels of ROS in Porcine Blastocysts

We investigated the intracellular levels of ROS in E-

Table 2. Effect of E-64 treatment for 24 h on preimplantation developmental competence in porcine embryos

Concentration (μM)	No. of embryos examined	No. (%) of embryos cleaved	No. (%) of blastocysts produced
0	143	128 (89.9±3.7)	57 (40.2±4.8)[a]
5	151	141 (92.9±2.8)	60 (40.2±5.0)[a]
10	153	137 (89.9±3.5)	47 (30.7±4.2)[b]
20	153	129 (84.2±4.9)	34 (22.3±4.8)[c]

Data are representation results in five independent experiments.
Data are the mean±S.D.
[a~c]Values with different superscripts within a column differ significantly (p<0.05).

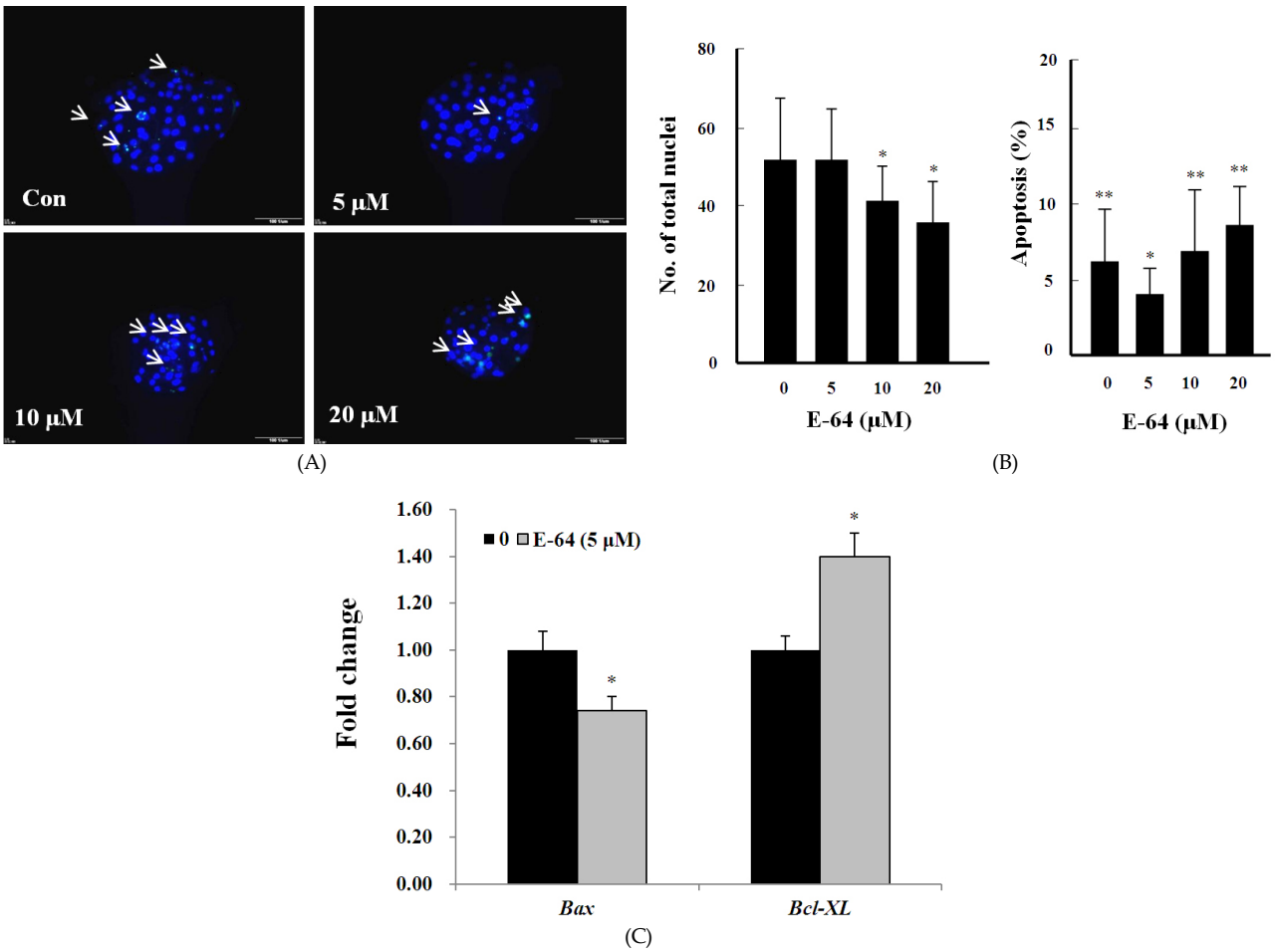
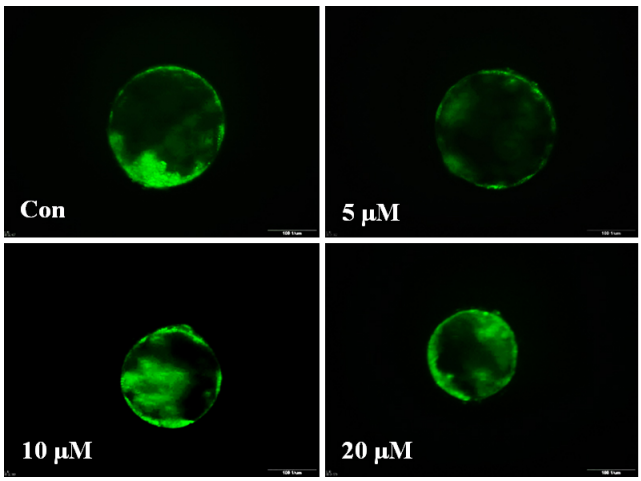
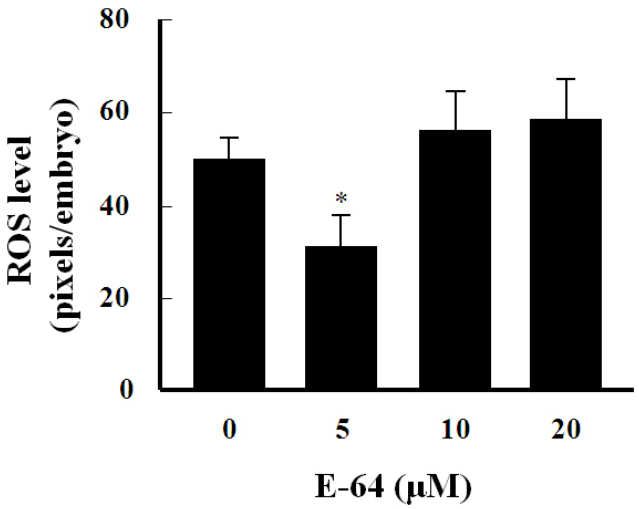


Fig. 2. Comparison of epifluorescent images (A), apoptosis index (B) and relative mRNA abundance of apoptotic genes (C) in porcine blastocysts derived from E-64 treated and non-treated groups. The chromatin contentis stained by DAPI (blue), fragmented DNA is labeled by the TUNEL reaction (green), and colocalization with DAPI appears sky-blue. Data are the mean±SD. Statistically significant differences are indicated by asterisks (p<0.05). Scale bars=100 μm.

64 treated blastocysts. As shown in Fig. 3A and 3B, intracellular levels of ROS did not differ significantly between the control and groups treated with 10 and 20 μM E-64. However, intracellular levels of ROS were significantly decreased in blastocysts derived from 5 μM E-64 treated embryos when compared with other gr-



(A)



(B)

Fig. 3. Comparison of antioxidant characteristics in porcine blastocysts derived from E-64 treatment groups. Fluorescence microscopy imaging of intracellular ROS expression (A) and ROS relative intensity (B) in porcine blastocysts derived from E-64 treatment. Data are the mean±SD. Statistically significant differences are indicated by asterisks (p<0.05). Scale bars=100 μm.

oups (p<0.05).

DISCUSSION

Lysosome is one of the cellular organelles, it is well known that the function of lysosome is to digest break down waste materials and cellular debris. Also, lysosomes contain many proteases, which plays an important role in the degradation of intracellular proteins (Dean, 1977). Sometimes lysosomal enzyme cathepsin B activity is increased in the cytoplasm, which is caused by leakage from lysosomes (Wyczalkowska and Paczek, 2012). Lysosomal disruption and cathepsin B redistribution to the cytoplasm is lead to oxidative stress and initiates apoptotic signaling (Yeung *et al*., 2006), which is involved in the apoptotic process of various cells such as thymocyte, blood and cancer cells (Sandes *et al*., 2007, Zhang and Li, 2012). Likewise, cathepsin B activity in the cytoplasm also appears in germ cells as well as in other cells (Oberle *et al*., 2010). A recent study by Balboula *et al*. (2010b) on bovine showed that E-64 treatment significantly improved the quality and developmental competence of bovine IVF embryos. The objective of this investigation was to elucidate the lysosomal apoptotic mechanisms utilized by E-64. The E-64 can irreversibly inhibit cathepsin B through the selective binding to the cysteine active site (Feng *et al*., 1996). In this study examined on the effects of E-64 on preimplantation developmental competence and quality in porcine embryos.

To investigate the effect of cathepsin B inhibition on preimplantation developmental competence in porcine embryos, E-64 was added to the porcine embryo for 24 h in culture medium. In this study showed that E-64 treatment may have effects on the quality and developmental competence of porcine IVF embryos. Moreover, the E-64 treatment showed that 5 μM E-64 significantly improved the developmental competence without any noticeable effect on cleavage rate. Otherwise, the treatment of E-64 was higher by 10 and 20 μM, no increased in the developmental competence of porcine IVF embryos was observed (Table 2). Consequently, 5 μM E-64 treatment was determined to be optimal concentration for efficient porcine embryo culture, because some cathepsin B activity is may necessary for general embryonic development, which is also resemblance to that of previous studies on mouse embryos (Afonso *et al*., 1997).

Apoptosis is important parameter of embryo quality in the pig, which may correlate with release of cytochrome *c*, caspase activation and DNA fragmentation in the individual blastomeres. Thus, fragmentation of cleaved embryos is associated with subsequent apoptosis (Hao *et al*., 2003). In this study, 5 μM E-64 treatment was shown to decrease the index of apoptosis in porcine blastocysts (Fig. 2B). Moreover, we examined the effect of E-64 on apoptosis related genes transcript level. In porcine blastocysts, we found that the expression of *Bcl-xL* increased in the 5 μM E-64 treated group, whereas expression of *Bax* decreased (Fig. 2C). The *Bcl-xL* is prevent the *Bax* induced caspase activation, caspase activation is essential for the *Bax* induced apoptosis (Kitanaka *et al*., 1997). These results suggest that the cathepsin B inhibition could modulate expression of

apoptotic genes. Therefore, the quality of porcine embryos may associate with the change of apoptotic pattern by E-64 treatment.

In the recent decades, measurement of ROS has become a useful tool for studying oxidative stress and for embryo quality evaluation (Lu and Gong, 2009). The ROS is strongly react with intracellular proteins, and may cause severe dysfunction such as DNA fragmentation (Goto *et al*., 1992). Inappropriate culture condition such as incomplete chemical compounds, physical transition and unstable CO_2 concentration is generate ROS in developing embryos. Many studies have been focused on overcoming these detrimental effects of ROS (Kitagawa *et al*., 2004, Koo *et al*., 2008). For example, supplementation of cysteine increased intracellular glutathione peroxidase activity. It has the protective role from apoptosis induced by ROS (Kayanoki *et al*., 1996). In this study, porcine embryos cultured with E-64 for 24 h has reduced the oxidative stress in the blastocyst stage (Fig. 3). We speculate that this may be due to the role of higher cysteine residues by cathepsin B inhibition, and it is reduced ROS level as shown in Fig. 3. Further studies are required to determine the reactive oxygen species involved and possible involvement of the anti-oxidation system related to cysteine residues in porcine embryos.

In conclusion, this study demonstrated that the porcine embryos on addition of 5 μM E-64 in culture medium showed higher developmental competence and total cell numbers. Furthermore, 5 μM E-64 treatment groups resulted in significantly lower apoptotic index than those found in other groups. Consistent with above results, decreased expression of *CTSB*, *Bax* and increased expression of *Bcl-xL* were observed in E-64 treated groups when compared to the control. Also, cathepsin B inhibitor improves the embryo culture environment by reducing the reactive oxygen species content. Therefore, we propose that the proper inhibition of cathepsin B improves the blastocyst quality, which may be related to not only the apoptosis reduction but also the oxidative stress reduction in porcine embryos.

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