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—Original Article—

A Cathepsin B Inhibitor, E-64, Improves the Preimplantation Development of Bovine Somatic Cell Nuclear Transfer Embryos

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Abstract. Bovine somatic cell nuclear transfer (SCNT) is an important and powerful tool for basic research and biomedical and agricultural applications, however, the efficiency of SCNT has remained extremely low. In this study, we investigated the effects of cathepsin B inhibitor (E-64) supplementation of culture medium on *in vitro* development of bovine SCNT embryos. We initially used three concentrations of E-64 (0.1, 0.5, 1.0 μM), among which 0.5 μM resulted in the highest rate of blastocysts production after *in vitro* fertilization (IVF), and was therefore used for further experiments. Blastocyst development of SCNT embryos in the E-64 treatment group also increased relative to the control. Moreover, the cryosurvival rates of IVF and SCNT blastocysts were increased in E-64 treatment groups when compared with the control. On the other hand, we found that IVF and SCNT blastocysts derived from E-64-treated groups had increased total cell numbers and decreased apoptotic nuclei. Furthermore, assessment of the expression of apoptosis-related genes (Bax and Bcl-xL) in bovine IVF and SCNT blastocysts treated with E-64 by real-time RT-PCR analysis revealed suppressed expression of the pro-apoptotic gene Bax and stimulated expression of the anti-apoptotic gene Bcl-xL. Taken together, these finding indicate that addition of E-64 to embryo culture medium may have important implications for improving developmental competence and preimplantation quality in bovine IVF and SCNT embryos.

Key words: Apoptosis, Blastocysts, Bovine, Cathepsin B inhibitor (E-64), Somatic cell nuclear transfer (SCNT)

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Somatic cell nuclear transfer (SCNT) has been successfully applied to animal reproduction in a variety of species. However, the efficiency of cloning remains low in most animals. Recent studies have shown that supplementation of culture medium with melatonin [1], phytohemagglutinin [2], proteasome inhibitor [3], histone deacetylase inhibitor [4], and vascular endothelial growth factor [5] enhanced developmental competence of SCNT embryos. Thus, culture condition is one of the critical factors for determining *in vitro* developmental competence of SCNT embryos.

Cathepsin B is a lysosomal cysteine protease that degrades intracellular proteins in lysosomes [6]. This activity can be attributed to its effects on the apoptotic pathway through activation of initiator caspases rather than executioner caspases [7]. Cathepsin B has also been shown to activate caspases indirectly via mitochondrial membrane degradation, leading to translocation of apoptosis-initiating components from mitochondria to cytoplasm [8]. E-64 is a very useful cysteine protease inhibitor of cathepsin B that is widely permeable in cells and tissues and has low toxicity [9]. According to Balboula *et al*. [10], bovine embryos cultured with E-64 promoted increased blastocyst formation. Furthermore, addition of E-64 to culture medium has been confirmed to increase total cell number and decrease TUNEL-positive nuclei in bovine IVF blastocysts.

The process of apoptotic cell death in bovine preimplantation embryos has been well described, and the occurrence of apoptosis in preimplantation embryos is considered one of the most important parameters for evaluation of embryo quality [11]. The TUNEL reaction is the most frequently used method for detection of apoptotic cells, which is accomplished by labeling of extensive oligonucleosomal DNA fragmentation generated by endogenous DNase activity during the apoptotic process [12]. Application of the TUNEL reaction assay for preimplantation embryos has confirmed apoptosis in the bovine embryo development [13]. Moreover, increased incidence of cell death is an important indicator of inadequate or suboptimal embryo culture conditions [14]. Furthermore, apoptosis increased occurrence of poor quality bovine embryos, which was related to higher and lower levels of expression of apoptosis-related genes, Bax and Bcl-xL, respectively, when compared with good quality embryos [15].

To the best of our knowledge, the effects of E-64 on the developmental potential of bovine SCNT embryos have not yet been reported. Therefore, the present study was conducted to investigate the effects of the addition of E-64 to *in vitro* culture (IVC) medium on the developmental ability and quality of bovine SCNT embryos. We also examined the expression of apoptosis-related genes in SCNT embryos with and without E-64 treatment.

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Materials and Methods

Chemicals

Unless otherwise noted, all chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA).

In vitro production of bovine embryos

In vitro maturation (IVM) of bovine oocytes was performed as described by Song *et al*. [16], with slight modification. Briefly, bovine ovaries were collected from a local slaughterhouse and transported to the laboratory in 0.9% saline at 25–30 C. Cumulus-oocyte complexes (COCs) were then aspirated from 3 to 6-mm follicles using a disposable 10-ml syringe with an 18-gauge needle. Aspirated COCs with at least three layers of compact cumulus cells and homogeneous cytoplasm were washed three times in Tyrode's lactate-N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (TL-HEPES) [1 mg/ml bovine serum albumin (BSA), low carbonate Tyrode-albumin-lactate-pyruvate (TALP)] [17]. Ten oocytes were matured in 50 µl of IVM medium in a 60-mm dish (Nunc, Roskilde, Denmark) under mineral oil for 20–22 h at 38.5 C under an atmosphere of 5% CO_2 in air. The medium used for oocyte maturation was TCM-199 (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco-BRL), 10 µg/ml pregnant mare serum gonadotropin (PMSG; Sigma), 0.6 mM cysteine, 0.2 mM Na-pyruvate and 1 µg/ml estradiol-17β. Following IVM, 15 oocytes were fertilized with frozen-thawed sperm at a concentration of 2×10^6 cells/ml in 50 µl of fertilization medium (Fert-TALP). When sperm were added to the fertilization drops, 2 µg/ml heparin, 20 µM penicillamine, 10 µM hypotaurine and 1 µM epinephrine (PHE) were also added. After 22 h of insemination, cumulus-enclosed oocytes were stripped using gentle pipetting and transferred to Charles Rosenkrans amino acid (CR1aa) medium containing 0.3% BSA, 1 × basal medium Eagle (BME) amino acids and 1 × minimum essential medium (MEM) nonessential amino acids for *in vitro* culture (IVC) [18]. After culture for three days, the cleaved embryos were further cultured in medium containing 50 µl of CR1aa (with 10% FBS) for four days at 38.5 C in 5% CO_2 in air. E-64 was added to the culture medium at different concentrations, according to the experiment design.

Somatic cell nuclear transfer

Experiments were conducted according to the Animal Care and Use Committee guidelines of the National Livestock Research Institute of Korea. Cell culture and assessment procedures have been described previously [19]. Bovine ear skin fibroblast (bESF) cells were used as donor cells for nuclear transfer. Bovine ear skin was surgically isolated, chopped into small pieces, and cultured in 100-mm culture dishes containing Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS. The cells were maintained at 37 C in 5% CO_2 for two weeks until they became confluent, after which the bESFs were passaged three times before use as a source of donor nuclei for SCNT. Mature oocytes were transferred to 500 µl of TL-HEPES supplemented with 0.1% hyaluronidase and were freed of cumulus cells by mechanical pipetting. The zonae pellucidae of oocytes were partially dissected using a fine glass needle. Oocyte manipulations such as enucleation and cell injection were performed using a micromanipulator (Narishige, Tokyo, Japan) equipped with an inverted microscope (Nikon, Tokyo, Japan). The medium used for the manipulation was TL-HEPES containing 7.5 µg/ml cytochalasin B. The first polar bodies and partial cytoplasm presumptively containing metaphase II chromosomes were removed together using a micropipette with an inner diameter of 20 µm. Successful enucleation was confirmed by Hoechst 33342 staining and visualization under ultraviolet light. Single cells were individually transferred to the perivitelline space of the recipient cytoplasts. The cell-cytoplast complexes (CCCs) were subsequently equilibrated in a 50-µl drop of cell fusion medium (FM) for 10–20 sec and then transferred to a fusion chamber filled with FM [0.3 M mannitol, 0.5 mM HEPES, 0.01% BSA, 0.1 mM $CaCl_2$ and 0.1 mM $MgCl_2$]. The CCCs were induced to fuse with a single direct-current pulse of 22 V applied for 40 µsec using a cell fusion generator (LF201, Nepa Gene, Chiba, Japan). All procedures were performed at room temperature. Reconstructed embryos without visible somatic cells 1 h after the fusion pulse were determined to be fused eggs. For activation, we used a modified method described by Su *et al*. [20]. Briefly, at 4 h after electrofusion, the fused eggs were activated with 5 µM ionomycin for 5 min, followed by treatment with 2.5 mM 6-dimethyl-aminopurine in CR1aa supplemented with 0.3% BSA for 3.5 h at 38.5 C under 5% CO_2 in air. The embryos were then cultured as described above.

Vitrification and warming

Cryopreservation was carried out by vitrification with a cryotop (Kitazato Supply, Fujinomiya, Japan) using a slightly modified version of the procedure described by Kuwayama [21]. Briefly, IVF- and SCNT-derived bovine blastocysts were transferred into equilibration solution (ES) consisting of 7.5% ethylene glycol (EG) and 7.5% dimethylsulfoxide (DMSO) in PBS supplemented with 20% FBS at room temperature for 5 min. Next, one or two blastocysts were transferred into vitrification solution (VS) consisting of 15% EG, 15% DMSO and 0.5 M sucrose dissolved in PBS containing 20% FBS. After 20 to 30 sec, the blastocysts were loaded into a cryotop and plunged into liquid nitrogen. The process from exposure in VS to plunging into liquid nitrogen was completed within 1 min. Vitrified blastocysts were then warmed by immersing the cryotop directly into warming solution (1.0 M sucrose dissolved in PBS containing 20% FBS) for 1 min, after which they were transferred to dilution solution (0.5 M sucrose dissolved in PBS containing 20% FBS) for 3 min, and then to dilution solution (0.25 M sucrose dissolved in PBS containing 20% FBS) for 5 min. Subsequently, blastocysts were incubated for 5 min in washing solution (PBS containing 20% FBS). Survival of vitrified-warmed IVF- and SCNT-derived blastocysts was determined according to re-expansion and hatched rates after 24 and 48 h of recovery in culture medium.

TUNEL assay

Apoptotic cells in blastocysts were detected using an *In Situ* Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany). IVF- and SCNT-derived blastocysts were washed three times with 0.1% PVP in PBS and then fixed in 4% (v/v) paraformaldehyde diluted in PBS for 1 h at room temperature. For membrane permeabilization, fixed embryos were incubated in PBS containing 0.1% (v/v) Triton

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

Gene	Primer sequence	Annealing temperature (C)	Product size (base pairs)
β-actin	F:CTCTTCCAGCCTTCCTTCCT R:GGGCAGTGATCTCTTTCTGC	60	156
Cathepsin B	F:GGTTGCAGACCGTACTCCAT R:GGAACTGCATCCAAAATGCT	60	246
Bax	F:TCTGACGGCAACTTCAACTG R:TGGGTGTCCCAAAGTAGGAG	60	246
Bcl-xL	F:GGTATTGGTGAGTCGGATCG R:AGAACCACACCAGCCACAGT	60	195

X-100 for 1 h at 4 C. Fixed embryos were preincubated in terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick end-labeling (TUNEL) reaction medium for 1 h at 38.5 C in the dark, washed and mounted on slides with mounting solution containing 1.5 μg/ml of 4′, 6-diamidino-2-phenylindole (DAPI; Vector Laboratories). DAPI-labeled or TUNEL-positive nuclei were subsequently observed under a fluorescence microscope (Olympus, Tokyo, Japan). Nuclei were recorded as positive for TUNEL labeling only when they showed light green fluorescence on the blue fluorescent background of DAPI. The person counting cells and apoptotic nuclei was unaware of the experimental group.

RNA isolation and real-time RT-PCR

Five blastocysts derived from IVF (day 7) and SCNT (day 6) embryos were collected, after which the mRNA from blastocysts was isolated using a Dynabeads mRNA Direct Kit (Invitrogen) and RNeasy Plus Micro Kit (Invitrogen) according to the manufacturer's protocol. The RNA was reversed transcribed in a 20-μl reaction mixture containing 8 μl RNA, 50 ng/μl random hexamers, 10 mM dNTP mix, 10X RT buffer, 25 mM $MgCl_2$, 0.1 M DTT, 40 U/μl RNaseOut and 200 U/μl SuperScript III RT. The reaction was carried out by subjecting the mixture to 65 C for 5 min, 50 C for 50 min, 85 C for 5 min and then cooling to 4 C, after which the cDNA was used as a template for PCR. Each sample was analyzed with β-actin as an internal control. Real-time RT-PCR was performed using an Applied Stratagene Mx3000P QPCR system (San Diego, CA, USA) in a final reaction volume of 20 μl with SYBR Green (Qiagen). All samples were quantified simultaneously during the same run with the housekeeping gene β-actin. The primers used for real-time RT-PCR are shown in Table 1. The amplification program was as follows: preincubation for fast-start polymerase activation at 95 C for 10 min, 45 amplification cycles of denaturation at 95 C for 40 sec and then acquisition of fluorescence. After the end of the last cycle, a melting curve was generated by initiating fluorescence acquisition at 72 C and taking measurements every 0.1 C until 95 C was reached.

Experimental designs

Four experiments were conducted in this study, and each experiment was replicated at least five times. In experiment 1, we evaluated the influence of E-64 on the developmental competence of bovine IVF (day 7) and SCNT (day 6) embryos. IVF-derived embryos were allocated randomly and cultured for 24 h in IVC medium containing 0.1, 0.5 or 1.0 μM E-64, after which they were incubated in freshly prepared IVC medium without E-64. SCNT-derived embryos were also grouped as described above and then cultured for 24 h in IVC medium containing 0.5 μM E-64, after which they were incubated in freshly prepared IVC medium without E-64. The cleavage status of the embryos was evaluated at day 2 of IVC. Blastocysts were examined on day 7 (IVF) and day 6 (SCNT) of embryo culture. In experiment 2, the cryosurvival rate was compared between the E-64-treated group and the untreated control group. In experiment 3, the effects of E-64 treatment on the apoptotic index of blastocysts derived from bovine IVF (day 7) and SCNT (day 6) embryos were examined. In experiment 4, the mRNA expression of cathepsin B and apoptosis-related genes in both sets of blastocysts derived from E-64 treatment was also investigated.

Statistical analysis

All experiments were repeated more than three times. All percentage data are presented as the means ± SD. The Student's *t*-test was used for comparison between two groups, and Duncan's multiple range tests were used for multiple comparisons. A probability value of $P<0.05$ was considered significant.

Results

E-64 treatment improves developmental ability in bovine IVF and SCNT embryos

As shown in Table 2, the developmental rates were significantly increased in the groups treated with 0.1 and 0.5 μM E-64 ($P<0.05$). The 0.5 μM E-64 treatment group showed the highest rate of IVF blastocysts production among the groups. Conversely, no difference was observed in cleavage rates among the treatment and nontreatment groups. In further experiments conducted to investigate the developmental competence of SCNT embryos, we used 0.5 μM E-64. Application of E-64 to the IVC medium also led to significantly increased SCNT-derived blastocyst production (46.9 ± 4.9 *vs*. 37.6 ± 3.5%, $P<0.05$; Table 3), although there was no significant difference in the cleavage rate between the control (83.5 ± 2.4%) and treatment group (84.9 ± 2.9%).

E-64 enhances the cryosurvival rate of bovine IVF and SCNT blastocysts

The *in vitro* survival and hatching rates of IVF- (day 7) and SCNT-

Table 2. Effect of the addition of E-64 on the developmental competence of bovine IVF embryos

E-64 (μM)	No. of embryos examined	No. (%) of embryos cleaved	No. (%) of blastocysts
0	299	256 (85.7 ± 3.8)	124 (41.7 ± 4.5)[a]
0.1	292	255 (87.3 ± 3.0)	136 (46.7 ± 4.3)[b]
0.5	295	258 (87.4 ± 1.8)	145 (49.2 ± 2.9)[b]
1.0	298	253 (84.8 ± 4.1)	119 (39.9 ± 4.6)[a]

Data are means ± SD. [a,b] Values are from ten replicates; different superscripts denote a significant difference compared with the other groups (P<0.05).

Table 3. Effect of the addition of E-64 on the developmental competence of bovine SCNT embryos

E-64 (μM)	No. of embryos examined	No. (%) of embryos cleaved	No. (%) of blastocysts
0	291	243 (83.5 ± 2.4)	109 (37.6 ± 3.5)[a]
0.5	293	249 (84.9 ± 2.9)	137 (46.9 ± 4.9)[b]

Data are means ± SD. [a,b] Values are from ten replicates; different superscripts denote a significant difference compared with the other group (P<0.05).

derived (day 6) bovine blastocysts after vitrification/warming are summarized in Table 4. All vitrified/warmed IVF- and SCNT-derived bovine blastocysts were recovered. The survival rates of vitrified/warmed IVF- and SCNT-derived bovine blastocysts in the E-64 treatment groups were significantly higher than in the nontreatment groups (87.9 ± 3.6 *vs*. 82.5 ± 5.8% and 77.6 ± 5.8 *vs*. 69.8 ± 4.4%, P<0.05). Furthermore, the hatching rates of vitrified/warmed IVF- and SCNT-derived bovine blastocysts in E-64 treatment groups were significantly higher than those of the nontreatment groups (56.8 ± 5.9 *vs*. 48.2 ± 4.6% and 46.4 ± 4.1 *vs*. 39.8 ± 5.0%, P<0.05).

Effect of E-64 on the quality of bovine IVF and SCNT blastocysts

As shown in Fig. 1, the total cell numbers in IVF and SCNT blastocysts derived from the E-64-treated group were significantly higher than those of the control group (144.4 ± 7.8 *vs*. 138.9 ± 6.9 and 130.6 ± 5.9 *vs*. 123.1 ± 6.2, P<0.05). The apoptotic indexes of the IVF and SCNT bovine blastocysts derived from the E-64-treated group were significantly lower than those of the control group (2.0 ± 0.9 *vs*. 2.7 ± 0.8% and 2.1 ± 0.8 *vs*. 3.2 ± 1.4%, P<0.05; Table 5).

Effect of E-64 on cathepsin B and apoptosis-related genes expression of bovine IVF and SCNT embryos

The relative expression levels of cathepsin B and apoptosis-related genes, Bax and Bcl-xL, were analyzed in IVF and SCNT blastocysts from the E-64-treated groups using real-time RT-PCR (Fig. 2). The expression of mRNA for cathepsin B in the E-64-treated groups was lower in IVF- and SCNT-derived blastocysts compared with the control group (P<0.05). The expression of Bax was significantly lower in IVF- and SCNT-derived blastocysts from E-64 treatment groups than in the control (P<0.05). Otherwise, the expression level of Bcl-xL was significantly higher in IVF- and SCNT-derived blastocysts from E-64 treatment groups than in the control (P<0.05).

Discussion

SCNT in domestic animals has been contributed to basic research in developmental biology, medicine, and agriculture. Although there have been great advancements in the production of SCNT embryos, defects are still known to influence blastocyst viability [22]. The defects were revealed to be abnormal epigenetic modifications such as DNA methylation and histone modifications in SCNT embryos [23, 24]. Cathepsins are generally involved in protein degradation and processing and have been implicated in a variety of cellular processes including apoptosis, angiogenesis, cell proliferation and invasion [25]. The action of cathepsin B secreted from cells is dependent on the pH, stress, and the embryo culture conditions [26]. Under normal culture conditions, cathepsin B within cells is regulated by cysteine protease inhibitors [27]. Cathepsin B, which is a lysosomal cysteine protease, is an important factor that degrades intracellular proteins in lysosomes [6]. Sireesha *et al*. [28] reported that lysosomal cysteine proteases may play a critical role during late preimplantation development in the golden hamster. Interestingly, inhibition of cathepsin B during IVM as well as IVC usually increased the quality and developmental competence of bovine embryos [10, 29]. This type of activity of cathepsin B could be attributed to its effects on the apoptotic pathway through activation of initiator caspases rather than executioner caspases [7]. It has also been reported that cathepsin B can activate caspases indirectly via mitochondrial membrane degradation, leading to translocation of apoptosis-initiating components from mitochondria to cytoplasm [8]. Furthermore, cathepsin B inhibitors have been shown to attenuate mitochondrial cytochrome *c* release in various cell types [30], leading to mitochondrial and cellular protection. In this study, we found that treatment with a cathepsin B inhibitor, E-64, improved developmental competence during production of bovine IVF and SCNT embryos. Thus, regulation of cathepsin B during *in vitro* culture periods may be a useful method for improving the yield of bovine embryos produced by IVF and SCNT.

A recent study by Balboula *et al*. [10] on bovine showed that E-64 (0.5 μM) treatment for 7 days after IVF significantly improved the quality and developmental competence of bovine IVF embryos. Their results also showed that 0.5 μM E-64 treatments for 24 h significantly decreased the activity of cathepsin B in cleaved bovine embryos. Moreover, the results indicated that inhibition of cathepsin B at an early stage of IVC might affect the developmental ability of IVF embryos by controlling the intracytoplasmic conditions, possibly reducing unnecessary proteolysis or signaling relating to the apoptotic pathway [10]. Based on this previous report, we treated embryos with 0.5 μM E-64 for 24 h after IVF or SCNT. Thus, we confirmed that E-64 treatments (0.1 and 0.5 μM) significantly improved the developmental competence without any noticeable effect on cleavage rate (Table 2). Accordingly, we infer that E-64 treatment may also affect the quality and developmental competence of bovine IVF or SCNT embryos. On the other hand, in the group receiving the 1.0 μM E-64 treatment, there was no significant difference in the developmental competence of bovine IVF embryos. There was an

Table 4. Effect of E-64 on cryosurvival of bovine IVF and SCNT embryos

Group	E-64 (μM)	No. of embryos cryopreserved	No. (%) of embryos survived	No. (%) of embryos hatched
IVF	0	100	82 (82.5 ± 5.8)[a]	48 (48.2 ± 4.6)[a]
	0.5	100	88 (87.9 ± 3.6)[b]	56 (56.8 ± 5.9)[b]
SCNT	0	80	56 (69.8 ± 4.4)[a]	32 (39.8 ± 5.0)[a]
	0.5	80	62 (77.6 ± 5.8)[b]	37 (46.4 ± 4.1)[b]

Data are means ± SD. [a,b] Values are from eight replicates; different superscripts denote a significant difference between the E-64 treatment and control groups in IVF and SCNT embryos (P<0.05).

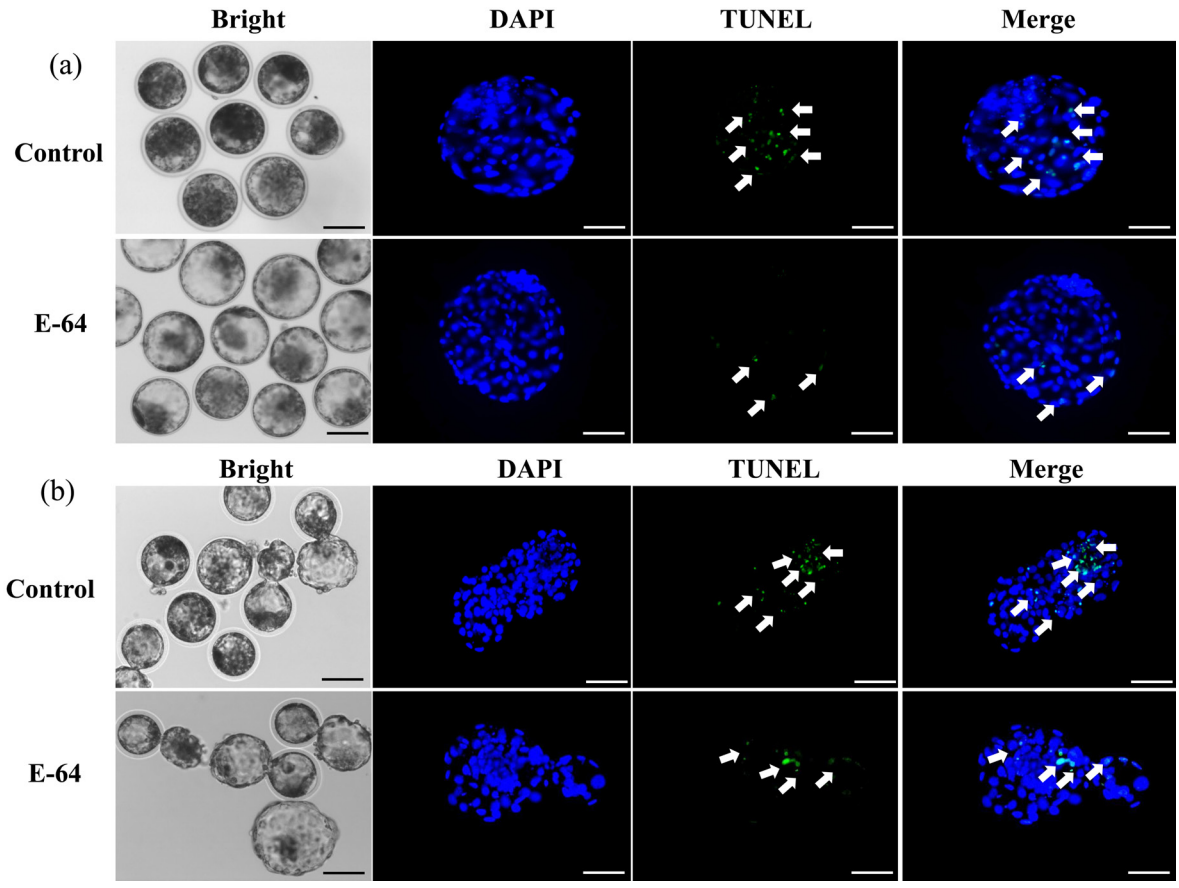

Fig. 1. Representative photographs and epifluorescent images of apoptotic patterns in bovine IVF- and SCNT-derived blastocysts. Total chromatin content was determined by staining with DAPI (blue), and fragmented DNA was labeled via terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL; green, white arrows). Merged images (light green color, white arrows) show TUNEL and DAPI signals. (a) Blastocysts derived from IVF. (b) Blastocysts derived from SCNT. Scale bar=100 μm.

Table 5. Effect of E-64 on blastomeres apoptosis of bovine IVF and SCNT embryos

Group	E-64 (μM)	No. of embryos examined	No. of cells		% of TUNEL-positive cells
			Total	TUNEL-positive	
IVF	0	28	138.9 ± 6.9[a]	3.8 ± 1.2[a]	2.7 ± 0.8[a]
	0.5	27	144.4 ± 7.8[b]	2.9 ± 1.3[b]	2.0 ± 0.9[b]
SCNT	0	26	123.1 ± 6.2[a]	3.9 ± 1.8[a]	3.2 ± 1.4[a]
	0.5	28	130.6 ± 5.9[b]	2.8 ± 1.1[b]	2.1 ± 0.8[b]

Data are means ± SD. [a,b] Values with different superscripts denote a significant difference between the E-64 treatment and control groups in IVF and SCNT embryos (P<0.05).

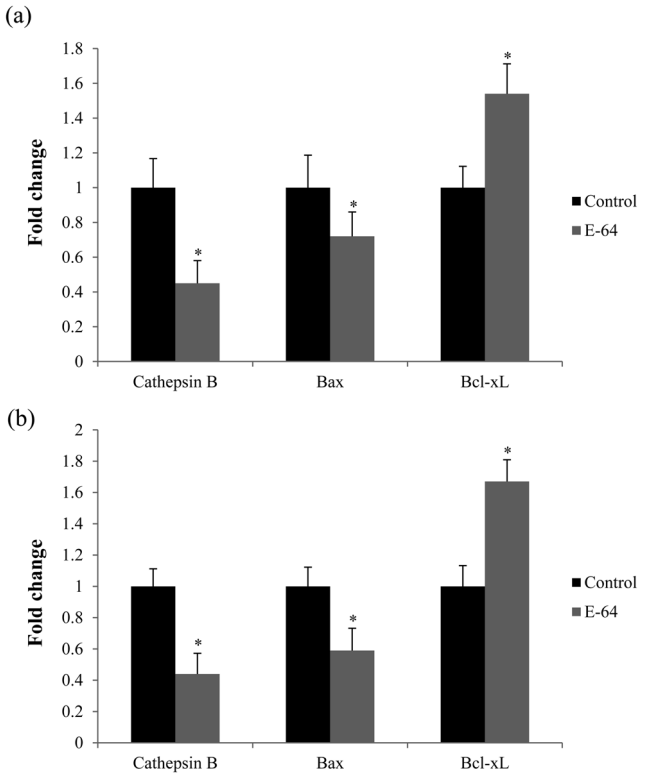

Fig. 2. Relative mRNA abundance of cathepsin B and apoptotic genes, Bax and Bcl-xL, in bovine IVF- and SCNT-derived blastocysts. (a) Blastocysts derived from IVF. (b) Blastocysts derived from SCNT. Data are the means ± SD. Statistically significant differences are indicated by asterisks (P<0.05).

existing dose-response window in bovine embryos.

The culture systems for embryonic development are also critical determinants for the successful development of cryopreserved SCNT embryos in a variety of mammalian species. An incomplete culture system for IVF-derived bovine embryos induces accumulation of intracellular lipid droplets, which are responsible for the high sensitivity of bovine embryos to cryopreservation [31]. Furthermore, bovine SCNT embryos are characterized by their higher chilling sensitivity and lower cryosurvival relative to their *in vitro* counterparts [32]. Due to SCNT-derived embryos have mechanical slit in their zonae pellucida, which the cryosurvival rates of SCNT blastocysts were low. Moreover, other reports have shown that the culture conditions of IVF and SCNT embryos are some of the most important factors affecting their ability to survive cryopreservation [33, 34]. This may result from the uptake of lipids from insufficient metabolism, high concentrations and variable distributions of cytoplasmic lipids with heterogeneous intracellular ice nucleation or lipid phase changes that contribute to the high sensitivity of the embryos to cryopreservation [35]. We found that the survival and hatching rates after cryopreservation of bovine IVF- and SCNT-derived blastocysts were improved by culture with E-64. However, no obvious difference in these embryos was observed under an optical microscope (Fig. 1). Thus, further studies including histochemical and ultrastructure examinations are needed to confirm the distributions of cytoplasmic lipids in bovine blastocysts.

In general, IVF- and SCNT-derived embryos have a lower total cell number and higher apoptotsis-positive cell number than *in vivo*-derived embryos in a variety of species [36]. One reason for this could be suboptimal culture conditions. The TUNEL assay enables the detection of apoptosis in mammalian embryos based on the commonly observed morphological features of apoptosis, including condensation or fragmentation of nuclei, or determination of the presence of specific DNA degradation products [37]. Hao *et al*. [38] reported that porcine IVF and SCNT embryos produced *in vitro* contained apoptotic cells, and that the incidences of DNA-fragmented nuclei were higher in SCNT embryos than in IVF embryos on day 6. We found that the total cell numbers in E-64 treatment groups were greater than those in nontreatment groups (P<0.05). On the other hand, the mean apoptotic-positive cell numbers in the E-64 treatment groups were lower than those in the nontreatment groups (P<0.05). Taken together, these results suggest that E-64 treatment improves the quality of IVF and SCNT embryos by regulating cathepsin B in the culture system.

To identify the cause of the reduced levels of apoptotic-positive cells, the relative expression levels of apoptosis-related genes were compared. The apoptotic pathway is achieved by the balanced expression of pro- and anti-apoptosis-related genes. Moreover, it has been reported that expressions of Bax and Bcl-xL genes are the critical determinants of both cell survival and death [39]. To confirm this mechanism, we measured the relative expression levels of apoptosis-related genes, Bax and Bcl-xL, and investigated whether E-64 reduced blastomere apoptosis by regulating apoptosis-related genes. Generally, Bax is increased in cells in which apoptotic death is promoted. This gene has been shown to play an important role in regulation of cell apoptosis during embryo development [40]. Furthermore, Bcl-xL plays a critical role in protecting cells from DNA damage [41]. In the present study, apoptosis increased in bovine IVF and SCNT embryos without E-64 treatment, which was related to the higher and lower levels of expression of Bax and Bcl-xL, respectively, when compared with the E-64-supplemented groups. Furthermore, cathepsin B mRNA transcripts were decreased in bovine IVF and SCNT embryos treated with E-64. This indicates that E-64 can effectively inhibit cathepsin B and that cathepsin B expression may be a negative correction between cathepsin B activity and quality in bovine IVF and SCNT embryos. Our results also indicated that E-64 might act as an apoptosis inhibitor in IVF and SCNT embryos against apoptosis and improve the quality of the resulting blastocysts. Thus, these results clearly support a promising role of cathepsin B inhibitor in improving blastocyst qualities of IVF and SCNT bovine embryos. However, further work will be necessary to completely understand the molecular mechanism by which E-64 regulates the expression of Bax and Bcl-xL in bovine IVF and SCNT embryos.

In conclusion, this study demonstrated that addition of E-64 to the culture medium for bovine IVF and SCNT embryos resulted in a higher rate of blastocyst production, higher rate of cryosurvival and higher total cell numbers. Furthermore, E-64 treatment groups had significantly fewer apoptotic-positive cells than the control groups. Consistent with the above results, decreased expression of Bax and increased expression of Bcl-xL were observed in E-64-treated groups when compared with the control. These findings suggest that E-64

could be used to improve the developmental competence and qualities of bovine embryos that may be used for SCNT animal research.

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