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 blastocyst 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.
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-hydroxyethylpiperezine-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% CO2 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 μM epinephrine (PHE) were also added. After 22 h of insemination, COCs were stripped using gentle pipetting and epinephrine (PHE) were also added. After 22 h of insemination, COCs were stripped using gentle pipetting.

Terminology of bovine embryos

The embryo stage 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 X-100 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.
Table 1. Primer sequences for real-time RT-PCR analysis

| Gene       | Primer sequence                  | Annealing temperature (°C) | Product size (base pairs) |
|------------|----------------------------------|---------------------------|---------------------------|
| β-actin    | F:CTCTTCCAGCCTCTTCTCTCTCTC       | 60                        | 156                       |
|            | R:GGGCAGTGATGTCCCTCTTCTGTCTC     |                           |                           |
| Cathepsin B| F:GGGTGCCACGCTGACTCCCAT          | 60                        | 246                       |
|            | R:GGAACGTGATCCCAAATGCT           |                           |                           |
| Bax        | F:TCGTACGGGACCTTCAACTG           | 60                        | 246                       |
|            | R:TGGGTGTCCCAAAGTGAAGG           |                           |                           |
| Bcl-xL     | F:GGTATTGGTGAGTCCGATCG           | 60                        | 195                       |
|            | R:AGAACCACACCCCAGGCCACAGT        |                           |                           |

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 cryo survival 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)      |
| 0.1       | 292                     | 255 (87.3 ± 3.0)            | 136 (46.7 ± 4.3)      |
| 0.5       | 295                     | 258 (87.4 ± 1.8)            | 145 (49.2 ± 2.9)      |
| 1.0       | 298                     | 253 (84.8 ± 4.1)            | 119 (39.9 ± 4.6)      |

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)      |
| 0.5       | 293                     | 249 (84.9 ± 2.9)            | 137 (46.9 ± 4.9)      |

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.
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 ± 6.9a | 3.8 ± 1.2a     | 2.7 ± 0.8a             |
|       | 0.5       | 27                      | 144.4 ± 7.8b | 2.9 ± 1.3b     | 2.0 ± 0.9b             |
| SCNT  | 0         | 26                      | 123.1 ± 6.2a | 3.9 ± 1.8a     | 3.2 ± 1.4a             |
|       | 0.5       | 28                      | 130.6 ± 5.9b | 2.8 ± 1.1b     | 2.1 ± 0.8b             |

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).
including histochemical and ultrastructure examinations are needed to observed under an optical microscope (Fig. 1). Thus, further studies with E-64. However, no obvious difference in these embryos was found that the survival and hatching rates after cryopreservation of bovine IVF- and SCNT-derived blastocysts were improved by culture 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 embryos treated with E-64 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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