—Full Paper—

Addition of Alpha-Tocopherol to Culture Medium Improves the Quality and Cryosurvival of Nuclear-Transferred Ovine Embryos

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Abstract. The aim of this study was to reconstruct and cryopreserve somatic cell nuclear transferred (SCNT) ovine embryos and evaluate the effect of α -tocopherol on blastocyst development and subsequent cryosurvival of the SCNT embryos. The α -tocopherol (100 μ g/ml) was added into culture medium for the SCNT embryos, the yield and total cell numbers of blastocysts were determined and the apoptosis incidences of the blastocysts were evaluated using the TUNEL assay. The blastocysts from the α -tocopherol and untreated groups were then frozen-thawed, and their cryosurvival was assessed by *in vitro* culture for 48 h. The results showed that there were no significant differences in blastocyst yield (26.3 vs. 22.3%) and total cell number (68.2 vs. 64.3) between the α -tocopherol and untreated groups. However, addition of α -tocopherol to the culture medium significantly decreased the apoptotic cell number (3.4 vs. 5.5) and significantly increased the cryosurvival of SCNT blastocysts (66.8 vs. 50.7%). In conclusion, addition of α -tocopherol to SCNT embryo culture medium was beneficial for improving embryo quality by decreasing the apoptotic blastocyst cell number and improving the tolerance of the embryos to cryopreservation.

Key words: Alpha-tocopherol, Apoptosis, Blastocyst, Cryopreservation, Nuclear transfer, Ovine

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Somatic cell nuclear transfer (SCNT) has been successfully applied for animal cloning in many species [1]. However, application of SCNT to animal production is still limited by the low development efficiency of SCNT embryos. The difficulties in cloning may be partly due to the *in vitro* culture system. For *in vitro* culture, an environment consisting of 5% CO₂ and 95% air is widely used. Under these conditions, the oxygen (O₂) concentration is about 20%, while the O₂ concentration under *in vivo* conditions is around 5–8% [2]. A higher oxygen concentration leads to an increased level of reactive oxygen species (ROS) and subsequent high level of oxidative stress in embryos, which is responsible for poor embryo quality [3]. Overproduction of ROS can have deleterious effects on the embryo by impairing the intracellular milieu and disturbing metabolism, which results in weakened cellular growth or apoptosis leading to embryo fragmentation [4, 5]. More frequent apoptosis occurs in blastocysts cultured *in vitro* than *in vivo* [6]. Embryos from mice [7], bovine [8] and ovine [9] cultured *in vitro* with a low (5%) O₂ concentration have been reported to show higher developmental rates than those cultured with 20% O₂, indicating that a high O₂ concentration during *in vitro* culture reduces developmental ability, perhaps due to increased accumulation of ROS in the cytoplasm of developing embryos [10].

Alpha-tocopherol (vitamin E) was first discovered as an anti-infertility factor in 1922 [11], and its deficiency affects embryo survival [12–14]. Wongsrikeao *et al.* [15] reported that supplementation of α -tocopherol during both recipient oocyte maturation and donor cell culture enhances blastocyst formation and

efficiently blocks DNA damage in transgenic SCNT bovine embryos. The blastocyst yield and number of cells in the blastocysts are improved in bovine and porcine embryos derived from *in vitro* fertilization and/or SCNT porcine embryos [16–19] when α -tocopherol is added to embryo culture medium. In contrast, Dalvit *et al.* [20] found that the presence of α -tocopherol or ascorbic acid in maturation medium failed to improve the percentage of blastocysts obtained in bovine embryos derived from *in vitro* fertilization and that the presence of the active form of α -tocopherol during maturation impairs the acquisition of oocyte developmental competence.

To our knowledge, no research has been conducted to verify whether α -tocopherol has an effect on the development and cryotolerance of SCNT ovine embryos. In the present study, SCNT ovine embryos were reconstructed, and the embryos were cultured at a high O₂ concentration (20%). A physiological concentration of α -tocopherol (100 μ g/ml) was added to the culture medium for SCNT ovine embryos, and the effect of α -tocopherol on developmental potential into blastocysts was investigated. In addition, we examined the survival of SCNT ovine blastocysts after cryopreservation because cryopreservation is often used as a measure to check the quality of *in vitro* produced embryos [21] and because cryopreservation of SCNT ovine embryos is useful for further studies and embryo transfer.

Materials and Methods

Reagents

All chemicals and media were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA) unless otherwise mentioned.

Preparation of donor cells

Ovine fibroblast cells were obtained from the ear of a 6-month-old female and were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco), 25 mM NaHCO₃ and 1% (v/v) nonessential amino acid solution (Gibco).

Preparation of oocytes

Ovine cumulus-oocyte complexes (COCs) were collected by aspiration of follicles in ovaries transported to the laboratory in normal saline at 20–25 °C within 4 h from the time of slaughter. The COCs were cultured in TCM-199 (Gibco) supplemented with 2.2 g/l NaHCO₃, 10% (v/v) FBS, 0.22 g/l sodium pyruvate, 5 µg/ml FSH, 1 µg/ml estradiol-17β and 10 ng/ml epithelial growth factor for 24 h. Cumulus cells were then removed by repeated pipetting in 0.1% (v/v) hyaluronidase in Hepes-buffered Ca²⁺-free PBS with 0.2 g/ml BSA, and oocytes with a first polar body were selected. The oocytes were enucleated with a micromanipulator (Olympus, Tokyo, Japan) in PBS supplemented with 5 µg/ml cytochalasin B and 10% FBS as the first polar body and adjacent cytoplasm presumably containing the metaphase-II chromosomes were extruded. After staining with 5 µg/ml bisbenzimidazole (Hoechst 33342) for 5 min, oocytes still containing DNA material were excluded.

Reconstruction and activation of SCNT embryos

A donor cell was inserted into the perivitelline space of an enucleated oocyte with a micromanipulator. The couplet was fused in fusion medium (0.28 mM mannitol, 0.1 mM MgCl₂, 0.05 mM CaCl₂) by placing each couplet between two stainless-steel electrodes attached to micromanipulators and subjecting them to two DC electrical pulses of 1.6 KV/cm for 10 µsec with an interval of 0.5 sec. The SCNT couplets were washed and cultured for 30 min in modified synthetic oviductal fluid (mSOF) until confirming successful fusion based on the absence of the donor cell in the perivitelline space. The SCNT embryos were cultured for 2 h in mSOF, and then chemical activation was induced by incubating them in mSOF containing 5 µM ionomycin for 5 min at 38.5 °C. After washing thoroughly in ionomycin-free mSOF, the SCNT embryos were further incubated for 4 h in mSOF supplemented with 2 mM of 6-dimethylaminopurine.

In vitro culture

Groups of 10 to 15 SCNT embryos were placed in 50 µl microdrops of mSOF supplemented with or without 100 µg/ml α-tocopherol, overlaid with mineral oil and cultured for 7 days at 38.5 °C in a humidified atmosphere of 5% CO₂ and 95% air. The culture medium was exchanged every 2 days. The stock solution of α-tocopherol was prepared in 95% ethanol and diluted in culture medium immediately before use. Development of embryos to the 2-cell and blastocyst stages was observed at 48 and 168 h of culture, respectively.

Detection of apoptosis by TUNEL assay

A TUNEL assay was performed to detect the presence of apoptotic cells in blastocysts using an in situ Cell Death Detection Kit (TMR red; Roche, Mannheim, Germany). At 168 h after SCNT,

the blastocysts were washed three times in PBS supplemented with 0.1% polyvinylpyrrolidone and fixed in 4% (v/v) paraformaldehyde/PBS solution for 24 h at room temperature. They were then permeabilized by incubation in 0.1% Triton X-100 in 0.1% citrate solution for 1 h and then incubated in TUNEL reaction medium for 1 h at 38.5 °C in the dark. After washing, they were transferred into 2 µg/ml of 4', 6-diamidino-2'-phenylindole dihydrochloride (DAPI; Roche)/PBS solution and mounted on slides with a ProLong Anti-fade Kit (Molecular Probes, Eugene, OR, USA). As positive controls for the TUNEL assay, fixed blastocysts were incubated in 50 U/ml RNase-Free DNase/PBS solution for 30 min at 38.5 °C in the dark before the assay. To determine the total cell and apoptotic cell numbers, DAPI-positive and TUNEL-positive cells in whole-mounted blastocysts were counted with a fluorescence microscope (Nikon, Tokyo, Japan), respectively.

Cryopreservation

The SCNT blastocysts harvested on Day 7 were exposed to 1.5 M ethylene glycol (EG) + 20% (v/v) FBS + 0.1 M sucrose in PBS at room temperature for 5 min and then loaded into 0.25 ml-plastic straws. The straws were transferred to an alcohol bath of a programmable freezer (ET-1; Fujihira, Tokyo, Japan) pre-cooled to -6.5 °C, held there for 5 min and then seeded by touching the straws with a forceps pre-cooled in liquid nitrogen. The straws were cooled to -35 °C at 0.3 °C/min and then plunged into liquid nitrogen. They remained frozen for 2 weeks until being thawed.

Thawing and culture of blastocysts

The straws were thawed by holding in air for 5 sec followed by transfer into water at 30 °C for 30 sec. The recovered blastocysts were washed three times in both 0.1 M sucrose in PBS and mSOF and then cultured in mSOF for 48 h at 38.5 °C in a humidified atmosphere of 5% CO₂ and 95% air. Blastocysts with the original volume of the blastocoel 24 h after culture and an enlarged blastocoel 48 h after culture were considered to have survived [22].

Statistical analysis

Random distributions of oocytes and embryos were created for each experimental group, and the experiments were replicated at least five times. For each replicate, the proportions of SCNT embryos reaching cleavage and developing to the blastocyst stage were transformed by an arcsine transformation. The transformed data were analyzed using one-way ANOVA and Scheffé's F-test, and cell numbers were compared using the Student's *t*-test.

Results

Effect of α-tocopherol on development and quality of SCNT embryos

As shown in Table 1, the cleavage rate and blastocyst yield of the SCNT embryos in α-tocopherol group (82.0 and 26.3%, respectively) showed no significant increase compared with the untreated group (84.8 and 22.3%, respectively).

As shown in Table 2 and Fig. 1, the mean total cell number of SCNT blastocysts cultured in the presence of α-tocopherol was 68.2, which was not significantly different from the untreated value

Table 1. Effect of α -tocopherol addition to embryo culture medium on the developmental ability of SCNT ovine embryos

α -tocopherol*	No. of SCNT embryos	No. of cleaved embryos (mean $\pm$ SE, %)	No. of blastocysts (mean $\pm$ SE, %)
+	429	82.0 $\pm$ 2.3	26.3 $\pm$ 2.9
–	437	84.8 $\pm$ 2.3	22.3 $\pm$ 3.6

*100 μ g/ml. The cleaved and blastocyst rates were calculated based on the total number of ovine oocytes in each treatment.

Table 2. Effects of α -tocopherol addition to culture medium on the total cell number and apoptosis index of SCNT ovine blastocysts

α -tocopherol*	No. of blastocysts	Total cell number (means $\pm$ SE)	No. of apoptotic blastomeres (means $\pm$ SE)	Apoptosis index (means $\pm$ SE)
+	30	68.2 $\pm$ 3.1	3.4 $\pm$ 0.5 ^a	0.049 $\pm$ 0.006 ^a
–	30	64.3 $\pm$ 3.8	5.5 $\pm$ 0.5 ^b	0.084 $\pm$ 0.009 ^b

*100 μ g/ml. Within each column, values with different superscripts differ significantly ($P < 0.05$). The apoptosis index was calculated by dividing the No. of apoptotic blastomeres by the total cell number.

Fig. 1. Detection of apoptotic cells by TUNEL assay. A: The nuclei in the blastocysts were stained using DAPI (blue color) and visualized under a fluorescein isothiocyanate (FITC) filter set. B: Fragmented nuclei of blastocyst were stained with a TUNEL kit (red color) and visualized under an FITC filter set. Scale bars=50 μ m.

Fig. 2. SCNT ovine blastocysts surviving cryopreservation. Scale bar=100 μ m.

Table 3. Effect of α -tocopherol addition to culture medium on cryosurvival of SCNT ovine blastocysts

α -tocopherol*	No. of thawed embryos	Survival at 24 h (mean $\pm$ SE, %)	Survival at 48 h (mean $\pm$ SE, %)
+	81	80.5 $\pm$ 4.0 ^a	66.8 $\pm$ 3.6 ^a
–	65	69.2 $\pm$ 1.7 ^b	50.7 $\pm$ 4.0 ^b

*100 μ g/ml. Within each column, values with different superscripts differ significantly ($P < 0.05$).

(64.3). However, the mean apoptotic cell number (3.4) and apoptosis index (0.049) in the α -tocopherol group were significantly lower than those of the untreated group (5.5 and 0.084, respectively).

Effect of α -tocopherol on cryosurvival of SCNT blastocysts

As shown in Table 3 and Fig. 2, the 24 and 48 h cryosurvival rates of the SCNT blastocysts in the α -tocopherol group were sig-

nificantly higher than those of the untreated group (80.5 vs. 69.2% and 66.8 vs. 50.7%, respectively).

Discussion

In the present study, although a slightly higher blastocyst yield (26.3 vs. 22.3%) and total cell number (68.2 vs. 64.3) were observed in SCNT ovine embryos cultured in mSOF with 100 μ g/

ml α -tocopherol compared with mSOF-alone, these differences were not statistically significant. It has been reported in SCNT porcine embryos that the yield and number are increased by addition of α -tocopherol to culture medium [18]. This is probably due to the different methods of embryo production employed and difference in animal species. However, the blastocyst yield was not sufficient to evaluate the quality of embryos. The total cell numbers of blastocysts were determined and the apoptosis incidences of blastocysts were evaluated using the TUNEL assay. The results showed that addition of α -tocopherol significantly decreased the number of apoptotic blastomeres, and this observation was consistent with previous reports [18, 19, 23]. Alpha-tocopherol is the most important antioxidant present in the ovary and follicular fluid. One function of α -tocopherol is to protect embryos against ROS, which can be produced by a high concentration of O_2 . When ROS production overwhelms antioxidant competence, oxidative stress occurs [24]. Oxidative stress seems to damage embryos by causing peroxidation of membrane phospholipids and altering most types of cellular molecules, such as lipids, proteins and nucleic acids [25]. Moreover, blastocoele fluid possessing H_2O_2 as a cytotoxic substance [26] can induce apoptosis in inner cell mass cells. Previous studies have shown [27, 28] that oxidants can indirectly induce apoptosis. Our results indicated that α -tocopherol might act as oxygen radical scavenger to protect SCNT embryos against oxidative stress and improve the quality of the resulting blastocysts.

Cryopreservation was used as another measure to check the quality of the SCNT ovine blastocysts. To the best of our knowledge, the effect of α -tocopherol on the freezing sensitivity of SCNT ovine embryos not been previously reported. This research showed that the embryos in the α -tocopherol group survived freezing significantly better than the untreated group. In the bovine, embryos produced *in vitro* were found to have higher chilling sensitivity than embryos obtained *in vivo*, and this phenomenon could mainly be due to suboptimal culture conditions *in vitro* [29, 30]. It has been suggested that the higher cryosurvival rate is attributable to the higher total cell number [21]. We speculate that the frequency of the apoptosis might also be related. In addition, α -tocopherol protects biological membranes from lipid peroxidation and alters components of cells [31, 32]; these improve the cryotolerance of embryos. Stabilization of erythrocyte membranes can improve their survival after cryopreservation [33].

It was suggested in this paper that the high concentration of O_2 had a negative effect on the development competence of SCNT ovine embryos. However, research on SCNT mouse embryos reconstructed with cumulus cells showed that a high concentration of O_2 is beneficial for development of SCNT embryos [34, 35]. A higher oxygen concentration is one of the possible causes of apoptosis. Apoptotic processes have both beneficial and detrimental effects, depending on their incidence. It can remove genetically abnormal cells from early embryos; in contrast, if the ratio of apoptotic cells is elevated, the presence of massive cell death could damage embryos, although the precise threshold frequency of apoptosis that is detrimental has not yet been determined in any species [36]. We discovered in this research that α -tocopherol improved blastocyst yield after cryopreservation and resistance to freezing in SCNT embryos probably by decreasing the number of apoptotic

cells in the embryos.

In conclusion, addition of 100 $\mu\text{g/ml}$ α -tocopherol to SCNT embryo culture medium was beneficial for decreasing the number of apoptotic cells in blastocysts, which might have improved tolerance of the blastocysts to cryopreservation. However, further studies are required to determine the optimum concentration of α -tocopherol for SCNT ovine embryos.

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