Effects of Recipient Oocyte and Embryo Culture System on Production of Hanwoo (Korean Native Cattle) Somatic Cell Nuclear Transferred Embryos

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ABSTRACT

This study was conducted to investigate an effective recipient oocyte and culture system for producing of Hanwoo (Korean native cattle) somatic cell nuclear transfer (SCNT) embryos. Hanwoo ear skin fibroblasts were used as donor cells. *In vitro* matured Hanwoo or Holstein oocytes were enucleated, and single donor cells were transferred into the perivitelline space of the enucleated oocytes. The couplets were subsequently fused and activated. The reconstructed embryos were cultured in a conventional or sequential culture system. In the former, embryos were cultured in CR2aa medium for eight days; in the latter, embryos were cultured in modified CR2aa-A (mCR2-A) for three days and then further cultured in modified CR2aa-B (mCR2-B) for five days. In the experiment with the recipient oocyte, the rate of embryo development to the blastocyst stage was significantly ($p<0.05$) higher in Hanwoo recipient oocytes than in Holstein ones (48.8% vs 38.9%). Blastocysts derived from Hanwoo recipient oocytes contained significantly ($p<0.05$) higher numbers of total cells than those derived from Holstein recipient oocytes (156.0±68.2 vs 134.7±54.8). There was no difference in the mean proportion of apoptotic cells in blastocysts between the sources of recipient oocytes. In the experiment with the embryo culture system, the blastocyst rate was somewhat higher in sequential system than in conventional system (50.0% vs 43.5%), though there was no significant difference. The numbers of total (160.0±69.0 vs 156.7±68.4) and apoptotic cells (14.0±10.4 vs 11.8±6.4) were not different between the culture systems. In conclusion, the present study demonstrated that Hanwoo recipient oocytes and the sequential culture system were more effective in supporting the production of Hanwoo SCNT embryos.

(Key words : Culture system, Korean native cattle, Nuclear transfer, Recipient oocyte)

INTRODUCTION

Successful somatic cell nuclear transfer (SCNT) has resulted in live domestic livestock clones, such as sheep (Wilmut *et al.*, 1997), cattle (Cibelli *et al.*, 1998), goats (Baguisi *et al.*, 1999), and pigs (Polejaeva *et al.*, 2000). However, the overall efficiency of SCNT is very low in comparison to *in vitro* fertilization (IVF). This is due to several factors, including the incompatible genetic backgrounds of donor cells and recipient oocytes, the synchronization of the cell cycle between recipient oocytes and donor cells, the nuclear transfer procedure *per se*, and embryo culture conditions (Dinnyes *et al.*, 2002; Heyman *et al.*, 2002; Renard *et al.*, 2002). Obstacles in the development of cloned embryos suggest that improvements are needed in terms of both the for embryo production procedures and the biological material used to clone embryos, such as recipient oocytes and donor cells.

The oocyte is a unique and complex cell that reprograms the donor nucleus; it is the one source of variation in cloning results (Miyoshi *et al.*, 2003). The recipient oocyte, however, has received much less attention. Previous studies that aimed to improve the development of cloned embryos using recipient oocytes have compared oocytes in metaphase I to those in metaphase II (Miyoshi *et al.*, 2001), oocytes derived from prepubertal versus adult animals (Betthauser *et al.*, 2000), oocytes matured rapidly versus slowly (Miyoshi *et al.*, 2002), the synchronization of oocytes prior to *in vitro* maturation by dibutyryl cAMP (Miyoshi *et al.*, 2002), the use of hybrid oocytes (Yang *et al.*, 2005), and the use of interbreed or interspecies oocytes (Gomez *et al.*, 2003). However, the effect of recipient oocytes on the developmental ability of Hanwoo (Korean native cattle) SCNT embryos has not been evaluated.

Optimal culture conditions are very important for the *in vitro* development of embryos. Under the non- or sub-optimal culture conditions, embryos show a delayed or abnormal expression of developmentally important genes and a lower developmental capacity in

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comparison to embryos derived *in vivo*. These results lead to elevated embryonic and fetal losses following embryo transfer to recipients (Niemann and Wrenzycki, 2000). Traditionally, the *in vitro* culture of IVF embryos has employed a conventional culture system, which a single medium is used for the entire culture period. For the culture system of SCNT embryos, conventional culture is chosen on the basis of the culture system used for IVF embryos. This culture system has also been used for culturing Hanwoo SCNT embryos.

Recently, a sequential culture system was developed for the *in vitro* development of mammalian embryos. This sequential culture system has the advantage of altering energy substrates and substrate concentrations, thus mimicking the change in environment experienced by developing embryos *in vivo* as they travel through the oviduct to the uterus (Swain *et al.*, 2001). This system has been shown to support the *in vitro* development of mammalian embryos, such as human and porcine (Ghandi *et al.*, 2001; Gardner *et al.*, 2002). However, it is unclear whether or not this sequential culture system is suitable for supporting the *in vitro* production of Hanwoo SCNT embryos or not.

The objectives of this study were to compare oocytes recovered from Holstein and Hanwoo as recipient cytoplasm, and compare conventional and sequential systems when used as embryo culture systems in the production of Hanwoo SCNT embryos.

MATERIALS AND METHODS

Preparation of Bovine Oocytes

Hanwoo and Holstein ovaries were collected from a local slaughterhouse and transported to the laboratory in a thermos containing saline at 30~35℃. Cumulus-oocyte complexes (COCs) were aspirated from 2 to 7 mm antral follicles using an 18-gauge needle and washed in Hepes-buffered TCM-199 (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco BRL). The COCs were incubated and matured in TCM-199 (Gibco BRL) supplemented with 10% FBS, 0.2 mM sodium pyruvate, 1 μg/ml follicle-stimulating hormone (FSH) (Folltropin-V; Bioniche, ON, Canada), 1 μg/ml estradiol 17-β, and 10 ng /ml epidermal growth factor (EGF; Sigma, St. Louis, MO, USA) at 39℃ in a humidified atmosphere of 5% CO_2 in air for 20 h. Hanwoo and Holstein COCs with a uniform ooplasm and at least one layer of compact cumulus cells were selected for the *in vitro* maturation. After maturation, the cumulus cells were removed by vortexing the COCs in Dulbecco's phosphate buffered saline (D-PBS) containing 0.1% hyaluronidase (Sigma) for 4 min. Mature oocytes with the first polar body and dense cytoplasm were selected as recipient cytoplasts.

Preparation of Somatic Cells

The somatic cells used in the current study were skin fibroblasts. Ear skin from an adult Hanwoo was cut into small pieces and cultured as tissue explants in Dulbecco's modified Eagle medium (DMEM; Gibco BRL) supplemented with 10% FBS and an antibiotic-antimycotic mixture (Gibco BRL) at 39℃ in a humidified atmosphere of 5% CO_2 in air. After seven days in culture, a fibroblast cell monolayer formed around the tissue explants. The explants were then removed, and the fibroblast cells were cultured to 100% of confluency. To passage cells, confluent cells were disaggregated by incubation in 0.05% trypsin-EDTA solution (Gibco BRL) and then allocated to tissue culture flasks. Fibroblast cells were grown to confluent passages 5 to 10 and used in the current experiment. These fibroblast cell monolayers were dissociated by trypsinization with 0.05% trypsin-EDTA solution, immediately prior to SCNT. Dissociated cells were suspended in D-PBS supplemented with 0.5% FBS.

Enucleation and Nuclear Transfer

Mature oocytes were enucleated in Hepes-buffered TCM-199 supplemented with 20% FBS and 50 μg/ml phytohemaglutinin (PHA; Sigma). The zona pellucida was partially dissected with a fine glass needle to create a slit near the first polar body. The first polar body and adjacent cytoplasm presumably containing the metaphase II chromosomes were extruded by squeezing with the needle. The enucleated oocytes were placed and incubated in Hepes-buffered TCM-199 supplemented with 20% FBS until SCNT occurred. A single cell with intact membrane was selected and transferred by micropipette into the perivitelline space of the enucleated oocyte through the slit that was made during enucleation.

Fusion and Activation

Karyoplast-cytoplast complexes were incubated in Zimmerman cell fusion medium (Zimmermann and Vienken, 1982) for equilibration, transferred into the petri dish containing Zimmerman cell fusion medium, and then manually oriented by fine electrical rods under light microscopy. Cell fusion was accomplished with a single DC pulse of 25 V/mm for 20 μs, delivered by an Embryonic cell fusion system (Fujihira Industry Co., Tokyo, Japan). Following electric stimulation, karyoplast-cytoplast complexes were incubated in Hepes-buffered TCM-199 supplemented with 20% FBS. After 30 min of electric stimulation, the fusion was then determined under a stereomicroscope.

After examination of cell fusion, all fused embryos

were immediately activated in CR2aa medium supplemented with 10 μM calcium ionophore (Sigma) for 5 min followed by CR2aa medium supplemented with 2 mM 6-dimethylaminopurine (DMAP; Sigma) for 3 h.

In Vitro Culture of Embryos

In the conventional culture system, activated bovine SCNT embryos were cultured for three days and in sets of 10 in 20-μl drops of CR2aa medium containing 3 mg/ml of fatty acid-free-bovine serum albumin (FAF-BSA; Sigma). Embryos were then switched to 20-μl drops of CR2aa medium containing 1.5 mg/ml BSA and 5% FBS, for an additional five days. In the sequential culture system, activated bovine SCNT embryos were cultured in sets of 10 in 20-μl drops of modified-CR2aa-A (mCR2-A) medium (CR2aa supplemented with 0.01 mM EDTA, 0.1 mM taurine and 0.5 mM glucose) containing 3 mg/ml FAF-BSA, for three days. Embryos were then switched to 20-μl drops of modified-CR2aa-B (mCR2-B) medium (CR2aa supplemented with 3.0 mM glucose) containing 1.5 mg/ml BSA and 5% FBS for an additional five days. Incubations for embryo culture were performed at 39℃ in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling (TUNEL) Assay

After 7 days of culture, the blastocysts were fixed in 4% paraformaldehyde in PBS and stored at 4℃ overnight. The blastocysts were washed twice in PBS containing 0.3% polyvinyl pyrrolidone (PVP-PBS). Fixed blastocysts were permeabilized with 0.5% Triton X-100 in PBS for 30 min at room temperature. The blastocysts were washed twice in PVP-PBS and incubated in 5-μl drops of TMR red-conjugated dUTP and deoxynucleotidyl transferase enzyme (Roche, Basel, Switzerland) in the dark for 1 h at 39℃. The blastocysts were washed for 5 min with PBS containing 0.5% Triton X-100 followed by PVP-PBS. For counterstaining, the blastocysts were incubated for 30 min in PBS containing 20 μg/ml bisbenzimide, at room temperature and in the dark. After counterstaining, blastocysts were washed twice in PVP-PBS, mounted onto glass slides with antifade solution, and examined under an inverted microscope equipped with epifluorescence.

Statistical Analysis

Data were subjected to a Generalized Linear Model procedure (PROC-GLM) of the Statistical Analysis System (SAS Institute, Cary, NC, USA). Differences among treatment means were determined by using Duncan's multiple range tests. All data were expressed as Least Square (LS) mean±SEM (Standard Error of the sample Mean). A probability of $p<0.05$ was considered statistically significant.

RESULTS

Development of Hanwoo SCNT Embryos according to Recipient Oocyte Source

The maturation rates to the metaphase II were about 70% both Hanwoo and Holstein recipient oocytes. There were no differences between the Hanwoo and Holstein recipient oocytes in term of fusion or cleavage rates, but the developmental rate to the blastocyst stage was significantly higher ($p<0.05$) in the Hanwoo recipient oocytes than the Holstein ones (48.8% vs. 38.9%) (Table 1). The total cell number of blastocysts derived from Hanwoo recipient oocytes (156.0±68.2) was significantly higher ($p<0.05$) than those derived from Holstein recipient oocytes (134.7±54.8). Between the sources of recipient oocytes, there was no difference in apoptosis rate (Table 2).

Development of Hanwoo SCNT Embryos according to Culture Systems

There was no difference between the conventional and sequential culture systems in terms of cleavage rate. The developmental rate to the blastocyst stage was higher in the sequential culture system than in the conventional culture system (50.0% vs. 43.5%), although there was no statistical significance therein (Table 3). The total cell number of blastocysts was not

Table 1. Effects of recipient oocytes on the development of Hanwoo SCNT embryos

Recipient oocytes	No. of oocytes NT	No. of oocytes fused	No.(%) of embryos cleaved*	No. of blastocysts (%)*		
				Day 6	Day 7	Day 8
Hanwoo	433	293 (67.7)	234 (79.9)	111 (37.9)	143 (48.8)[a]	146 (49.8)[a]
Holstein	335	216 (64.5)	178 (82.4)	64 (29.6)	84 (38.9)[b]	84 (38.9)[b]

[ab] Significant differences within the same column ($p<0.05$).
* Percentages were calculated based on the number of fused oocytes.

Table 2. Effects of recipient oocytes on the cell number and apoptosis of Hanwoo SCNT blastocysts

Recipient oocytes	No. of blastocysts examined	Cell number	Apoptotic cell number	Apoptotic cell (%)
Hanwoo	29	156.0±68.2[a]	10.9±8.7	6.5±4.6
Holstein	18	134.7±54.8[b]	8.2±8.0	6.0±4.8

[ab] Significant differences within the same column ($p<0.05$).

Table 3. Effects of culture system on the development of cloned bovine embryos with Hanwoo recipient oocytes*

Culture system	No. of embryos cultured	No.(%) of embryos cleaved	No. of blastocysts (%)		
			Day 6	Day 7	Day 8
Conventional	147	124 (84.4)	51 (34.7)	64 (43.5)	65 (44.2)
Sequential	154	131 (85.1)	53 (34.3)	77 (50.0)	79 (51.3)

* Hanwoo recipient oocytes were used for this experiment.

Table 4. Effects of culture system on the cell number and apoptosis of cloned bovine blastocysts with Hanwoo recipient oocytes*

Culture system	No. of blastocysts examined	Cell number	Apoptotic cell number	Apoptotic cell (%)
Conventional	18	156.7±68.4	11.8±6.4	6.3±3.5
Sequential	13	160.0±69.0	14.0±10.4	8.0±6.3

* Hanwoo recipient oocytes were used for this experiment.

different between the conventional and sequential culture systems (156.7±68.4 vs. 160.0±69.0). There was also no difference between the culture systems in terms of apoptosis rate (Table 4).

DISCUSSION

This study was carried out to investigate optimal recipient oocytes and an embryo culture system for the effective production of Hanwoo SCNT embryos.

There have been a few studies about the recipient oocyte on the pre-implantation development of SCNT embryos, despite the fact that the fundamental importance of oocyte cytoplasmic factors in reprogramming of transferred nuclei and early embryonic development has been well recognized (Chastant *et al.*, 1996; Fulka *et al.*, 2001; Gandolfi & Gandolfi, 2001). Previous studies of bovine SCNT were mainly focused on the maturation conditions and activation of oocyte (Miyoshi *et al.*, 2003), the maturity of the cattle as used for oocyte recipients (Salamone *et al.*, 2001) and the size of the follicle containing the oocyte (Piedrahita *et al.*, 2002). More recently, studies of interbreed recipient oocytes (Hiendleder *et al.*, 2004) and hybrid recipient oocytes derived from Holstein and Chinese Yellow cattle (Yang *et al.*, 2005) have been undertaken.

Although Roh and Yoon (2001) demonstrated the production of Hanwoo SCNT embryos using Holstein oocytes, there has been no comparative study of the Hanwoo and Holstein oocytes with respect to the development of Hanwoo SCNT embryos. In the current study, we compared the efficiency of the pre-implantation development of Hanwoo SCNT embryos reconstructed with Hanwoo and Holstein oocytes that matured *in vitro*. Our results showed that the use of Holstein recipient oocytes significantly reduced the *in vitro* development of Hanwoo SCNT embryos when compared to oocytes derived from Hanwoo. We assume that the low embryo development of Holstein recipient oocytes after Hanwoo SCNT in the current study was likely due to two factors. The first is the difference in recipient oocyte strain in the process of early nuclear reprogramming; after all, recipient oocyte strain-dependent differences in the ability to support SCNT embryo development have been reported in mice and cows (Gao *et al.*, 2004; Yang *et al.*, 2005). The second is the age of the oocyte donor animal. Generally, Holstein, which is traditionally a dairy breed are slaughtered at an older age than are Hanwoo, which is traditionally a beef breed. Decreased fertilization and bl-

astocyst rates of oocytes have been correlated with increasing age, in both humans and mice (Fujino *et al.*, 1996; Jannay and Menezo, 1996) and age-related declines in oocyte developmental competence have been linked to ooplasmic mitochondrial dysfunction (Thouas *et al.*, 2005). Mitochondria are the major source of an oocyte's energy supply, and they play a pivotal role in controlling the local intracellular pH by clustering around the spindle, conditioning the protein function and cytoskeletal organization, and regulating intracellular calcium homeostasis (Van Blerkom *et al.*, 2002).

Sub-optimal culture conditions for SCNT embryos are known to cause incomplete or inadequate reprogramming of donor nuclei, resulting in low rates of blastocyst formation and pregnancy (Han *et al.*, 2003). Culture media for IVF embryos have been greatly improved so that transferable embryos are routinely obtained from immature oocytes (Lee and Fukui, 1996). Conventional embryo culture systems that are currently used are typically static with a single medium used for the entire culture period; today, however, a new generation of sequential culture media has been developed. The design of such media focuses on the dynamics related to embryo physiology and metabolism, and the reduction of intracellular stress. Two media, G1 and G2, were therefore formulated for pre- and post-compaction embryos (Gardner, 1994; Barnes *et al.*, 1995). These media can support the development of viable blastocysts from many species, including those from humans, pigs and cows. (Ghandi *et al.*, 2001; Gardner *et al.*, 2002; Lane *et al.*, 2003). In the current study, the CR2aa medium was modified to mCR2-A and mCR2-B. On the basis of the CR2aa medium, mCR2-A was supplemented with 0.01 mM EDTA, 0.1 mM taurine, and 0.5 mM glucose; mCR2-B was supplemented with 3.0 mM glucose. Our results show that the sequential culture system of mCR2-A/mCR2-B supports the *in vitro* development of SCNT bovine embryos somewhat better than those cultured in the conventional single culture system of CR2aa, although there is no statistical significance therein. EDTA is an inhibitor of glycolytic kinases that helps prevent aberrant levels of glycolysis (Gardner *et al.*, 2000). EDTA has been shown to stimulate the cleavage stage embryo of both mice and cows (Gardner and Lane, 1996; Gardner *et al.*, 2000); however, the addition of EDTA to embryos from the eight-cell stage onward is not recommended, because continued exposure to EDTA negatively impacts blastocyst development and quality (Gardner *et al.*, 2000). Taurine is found in high concentrations in female reproductive tract fluid, and it serves as an antioxidant, chelating agent and osmolyte; it has been found to exert a positive effect on embryo development *in vitro* in mice, and cows (Liu and Foote, 1995; Spindle, 1995). Glucose has been used in embryo culture media, as a metabolite; however, it is known that glucose has an inhibitory effect on the development of preimplantation embryo in culture. On the basis of this conception, glucose was not added to the CR2aa medium in the culture of bovine embryos. On the other hand, it has been reported that glucose alone (i.e., without the presence of phosphate) showed no detrimental effect on the development of bovine embryos (Lim *et al.*, 2003). In the current experiment, we decided to add a concentration of EDTA, taurine, and glucose to the mCR2-A and mCR2-B media on the basis of the G1 and G2 media.

In conclusion, the use of Hanwoo recipient oocytes as a recipient cytoplasm and the sequential culture system as an embryo culture system improved the *in vitro* production of Hanwoo nuclear transfer embryos. To better enable the commercial application of these results, however, more in-depth study of embryo transfers is needed.

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