Viability of Somatic Cell Nuclear Transfer Embryos following Embryo Transfer in Korean Native Striped Cattle (*Bos namadicus* Falconer, Chikso)

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ABSTRACT

This study was conducted to examine the viability of Korean native striped cattle (*Bos namadicus* Falconer, Chikso) clone embryos after embryo transfer. Chikso somatic cell nuclear transfer (SCNT) embryos were produced by fusion of ear skin cells derived from a female Chikso with enucleated oocytes matured *in vitro* for 18-24 hr. After *in vitro* culture of SCNT embryos for 7 to 8 days, fresh or vitrified blastocysts derived from SCNT were transferred into a uterine horn of recipient cows. Fifteen of total 43 recipients were pregnant at Day 50 and 4 recipients were maintained to term. Three IVF-derived calves and 1 clone Chikso calf were born. Pregnancy rate was higher when fresh embryos were transferred to recipients compared to vitrified embryos, but development to term was not different between both groups. The clone Chikso calf died at 5 days after birth due to the fullness of amniotic fluid in rumen and the infection of umbilical cord. The result of the present study shows that clone Chikso calf can produced from the embryo transfer of SCNT embryos, however, solution of abortion problem is necessary to improve the cloning efficiency.

(Key words : SCNT, Embryo transfer, Vitrification, Viability, Chikso)

INTRODUCTION

Somatic cell nuclear transfer (SCNT) technique is a valuable tool for proliferating of genetically identified clone animals. Since the production of a cloned lamb from SCNT (Wilmut *et al.*, 1997), various species of animals have been cloned (Kato *et al.*, 1998; Wakayama *et al.*, 1998; Baguisi *et al.*, 1999; Wells *et al.*, 1999; Polejaeva *et al.*, 2000; Shin *et al.*, 2002; Galli *et al.*, 2003; Woods *et al.*, 2003; Lee *et al.*, 2005), Transgenic clone animals can be also produced by transfer of genetically modified somatic cells (Schnieke *et al.*, 1997; Cibelli *et al.*, 1998; Lai *et al.*, 2002).

Nuclear transfer (NT) technique has been also used for the proliferation of an endangered animal species (Lanza *et al.*, 2000; Loi *et al.*, 2001,). These trials have been conducted by Interspecies nuclear transfer which the donor cell derived from an endangered animal species was transferred in to the recipient cytoplasm derived from livestock animals (Dominko *et al.*, 1999; Loi *et al.*, 2001).

Though Chikso is not an endangered species, the population of them is very low. Thus, nuclear transfer technique may also be a useful tool for the proliferation of Chikso. In the present study, we examined the cloning efficiency of Korean native striped cattle (*Bos namadicus* Falconer, Chikso) by SCNT.

MATERIALS AND METHODS

Oocyte Collection and *In Vitro* Maturation (IVM)

Bovine follicular oocytes were obtained by aspirating follicles collected from slaughterhouse. Cumulus-oocyte complexes (COCs) were placed into a 50 μl droplet of maturation medium under light mineral oil (Sigma, St. Louis, MO, USA) and cultured at 39℃, 5% CO_2 in air for 18~20 hr. The maturation medium was composed of TCM-199 with Earle's salts (Gibco-BRL, Grand-Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL), 0.2 mM Na-pyruvate (Sigma), 50 μg/ml gentamicin (Gibco-BRL), 0.02U/ml FSH (Sigma) and 1 μg/ml estradiol-17 (Sigma). After maturation, cumulus cells of COCs were removed by vor-

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texing in 300IU/ml hyaluronidase (Sigma), and oocytes that extruded the first polar body were selected for recipient cells.

In Vitro Fertilization (IVF)

After IVM, the oocytes were fertilized with frozen-thawed sperm of the Korean native cattle at the concentration of 2×10^6 sperm/ml in 50 μl of BO medium (Brackett and Oliphant, 1975) supplemented with 5 mM caffeine (Sigma), 10 μg/ml heparin (Sigma) and 3 μg/ml of BSA. After 12~20 hr of insemination, COCs were transferred into 50 μl of TCM-199 supplemented with 3 mg/ml BSA and cultured for 32~40 hr. After then, cumulus cells were removed by vortexing for 2 min, and cleaved embryos were cultured in 50 μl drops of CR1aa medium supplemented with 10% FBS under light mineral oil (Sigma) at 39℃, 5% CO_2 in air for further 5 to 7 days.

Nuclear Transfer

Adult ear skin cells were obtained from a 3-year-old Korean native striped female cattle (*Bos namadicus* Falconer, Chikso) according to the previous study (Choi *et al.*, 2004). Micromanipulation procedures were performed in Hepes-buffered TCM-199 containing 5 μg/ml cytochalasin B (Sigma) and 3 mg/ml BSA. After maturation, cumulus cells of COCs were removed by vortexing in 300 IU/ml hyaluronidase (Sigma), and oocytes that extruded the first polar body were selected for recipient cells. Oocytes were enucleated by removing the first polar body and the metaphase II (MII) chromosome mass, which were protruded after treatment with 0.4 μg/ml demecolcine for 40 min. A single donor cell was injected into the perivitelline space of an enucleated oocyte through the same slit in the zona pellucida as made during enucleation.

Reconstituted oocytes were placed into a fusion chamber consisting of two wires, 0.5 mm apart, overlaid with 0.3 M mannitol containing 0.1 mM $MgSO_4$, 0.5 mM $CaCl_2$ and 0.05 mg/ml BSA. A single direct current (DC) pulse of 1.5 kV/cm for 30 μsec was applied to the chamber using a BTX Electro Cell Manipulator 200 (BTX, San Diego, CA). After fusion treatment, the reconstituted eggs were placed in TCM-199 containing 3 mg/ml BSA, and checked for fusion. Fused eggs were activated by exposing to 10 μM calcium ionophore (A23187, Sigma) for 5 min at 39℃, followed by treatment with 2 mM of 6-dimethylaminopurine (6-DMAP, Sigma) in CR1aa supplemented with 3 mg/ml BSA for 4 hr.

After activation, the embryos were cultured in 50 μl drop of CR1aa supplemented with 3 mg/ml BSA at 39 ℃, 5% CO_2 in air. After 2 days of culture, cleaved embryos were transferred into CR1aa supplemented with 10% FBS and further cultured at 39℃, 5% CO_2 in air for 5~7 days. NT embryos (NTs) developed to the blastocyst stage were served to the embryo transfer or vitrification.

Vitrification of Embryos

Vitrification of embryos was performed as described by Kwon *et al.* (2007). Briefly, blastocysts were equilibrated stepwise with vitrification solution (VS) 3, 2 and 1 for 5, 5 and 1 min, respectively, before being load into a straw. VS 1 was composed by 20% (v/v) glycerol, 20% (v/v) ethylene glycol, 3/8 M sucrose, 3/8 M dextrose, and 20% FBS (Gibco) in Dulbecco's PBS (D-PBS). VS 2 and 3 were made by 50% and 75% dilution of VS I with D-PBS, respectively. During last equilibration, straws were plunged into liquid nitrogen.

Before embryo transfer, vitrified embryos were thawed in 37℃ water bath, and washed in D-PBS supplemented with 0.5 and 0.25 M sucrose for each 5 min to dilute the cryoprotectants. Embryos were then washed in D-PBD containing 20% FBS, transferred and cultured in CR1aa containing 10% FBS at 39℃, 5% CO_2 in air for 4~6 hr up to embryo transfer.

Preparation of Recipient Cows

Recipient cows were prepared by hormonal estrus induction or natural estrus induction. For hormonal estrus induction, cows at Day 6-16 of estrus cycle were injected by 0.25 mg GnRH and followed by injection of 30 mg $PGF_{2\alpha}$ 7 days later. After 48 hr of $PGF_{2\alpha}$ injection, estrus cows were further injected by 0.25 mg GnRH and used for embryo transfer at Day 7~8 of estrus. Natural estrus cows were also used for embryo transfer at Day 7~8 of estrus. Recipient cows were checked for normal corpus leutium by rectal palpation before embryo transfer.

Embryo Transfer and Pregnancy Diagnosis

One or two blastocysts were loaded into 0.25 ml straw with D-PBS supplement with 20% FBS and culture in straw incubator at 37℃ for 1~2 hr up to embryo transfer. Two NTs or each 1 embryo derived from NT and IVF were transferred to one uterine horn of 43 recipient cows from 2 different farms. Pregnancy diagnosis was carried out by rectal examination or ultrasonography about 50 days after embryo transfer.

Microsatellite Assay

Genomic DNAs were extracted by genomic DNA extraction kit (TOYOBO MagExtractor NPK-101, Japan) from donor cells and bloods of cloned calf, donor and recipient cattle. Microsatellite assay was carried out by 5 bovine satellite markers; BM1824, TGLA122, ETH225, INRA023, and ETH10 (Vaiman and Mercier, 1994). Microsatellite primer sequences used in this study were shown in Table 1. Standard PCR conditions were as fo-

Table 1. Sequence specific primers used for microsatellite assay

Marker	PCR primer sequence
BM1824	Forward : 5'-gagcaaggtgtttttccaatc-3' Reverse : 5'-cattctccaactgcttccttg-3'
ETH10	Forward : 5'-gttcaggactggccctgctaaca-3' Reverse : 5'-cctccagcccactttctcttctc-3'
ETH225	Forward : 5'-gatcaccttgccactatttcct-3' Reverse : 5'-acatgacagccagctgctact-3'
INRA023	Forward : 5'-gagtagagctacaagataaacttc-3' Reverse : 5'-taactacagggtgttagatgaactca-3'
TGLA122	Forward : 5'-ccctcctccaggtaaatcagc-3' Reverse : 5'-aatcacatggcaaataagtacatac-3'

llows: pre-denature for 5 min at 95℃, followed by 30 cycles of denaturation for 30 sec at 95℃, annealing for 30 sec for 56℃, extension for 30 sec at 72℃, and post-extension for 5 min 72℃ and stored at 4℃ until analysis. Microsatellite PCR products were analyzed on ABI 3130 (Applied Biosystems, Foster City, CA, USA) using Genemapper (V 3.7) software.

Statistical Analysis

Embryo transfer data were analyzed by Chi-square test.

RESULTS

Two NTs or each 1 NT and 1 IVF embryos were transferred to total 43 heads of recipient. Of these recipients, 15 heads (34.9%) were pregnant at Day 50 after embryo transfer (Table 2~4). Eleven-one heads (73.3%) of pregnant recipients aborted at Day 57 to 246 of pregnancy, 4 heads allowed full term development.

Pregnancy rate of recipients which received fresh embryos at Day 50 after embryo transfer was higher than that of recipient which received frozen-thawed embryos (50% vs 21.7%, $p<0.05$), however, there was no difference in birth rate between both groups (Table 3).

High abortion rate (90.0%, 10/11) was shown in pregnant recipients that received NTs only compared to pregnant recipients that received NTs with IVF embryos (25.0%, 1/4). Only one female calf (9.1%) was born from a recipient received NTs at D 291 by Caesarian operation (Fig. 1), while 3 male calves (75.0 %) were born from 3 recipients received each 1 NT embryo and 1 IVF embryo by natural parturition (Table 4). These male calves were derived from IVF embryos.

The female calf derived from a recipient that was transferred NTs only (calf 1) was analyzed by microsatellite assay, and the result was shown in Table 5. Microsatellite assay result confirmed that calf 1 was NT-derived Chikso clone.

Clone Chikso calf died at Day 5 of birth, which may due to the excessive drinking of amniotic fluid into the reticulum and umbilical vessel infection (not shown).

DISCUSSION

There are many problems to solve including low efficiency of somatic cell nuclear transfer procedure and high frequencies of abortion, stillbirth, abnormality, large offspring syndrome and postnatal death for successful cloning in mammals (Shiga *et al.*, 1999; Wells *et*

Table 2. Embryo transfer results of bovine nuclear transfer (NT) embryos

Farm	No. of recipients	No. (%) of pregnant at D50	No. (%) of abortion	No. (%) of parturition	Note
A	21	6(28.6)	3(50.0)	3(50.0)	3♂
B	22	9(40.9)	8(88.9)	1(11.1)	♀, died 5d after born
Total	43	15(34.9)	11(73.3)	4(26.7)	

Table 3. Embryo transfer results of fresh and vitrified bovine nuclear transfer (NT) embryos

Treatment	No. of recipients	No. (%) of pregnant at D50	No. (%) of abortion	No. (%) of parturition	Note
Fresh	20	10(50.0)[a]	8(80.0)	2(20.0)	2♂
Vitrified	23	5(21.7)[b]	3(60.0)	2(40.0)	1♂ & 1♀

[a,b] Values with different superscript in the same column differ ($p<0.05$).

Table 4. Embryo transfer results following transfer methods

Treatment*	No. of recipients	No. (%) of pregnant at D50	No. (%) of abortion	No. (%) of parturition	Note
NT	33	11(33.3)	10(90.9)	1(9.1)	♀
NT+IVF	10	4(40.0)	1(25.0)	3(75.0)	3♂

* NT, transfer of 2 nuclear transfer (NT) embryos; NT+IVF, transfer of 1 NT and 1 IVF embryos.

Table 5. Microsatellite analysis of donor cells and blood, recipient and offspring

Marker	Donor cell	Donor blood	Calf 1 blood	Recipient blood
BM1824	181/183	181/183	181/183	181/181
TGLA122	151/181	151/181	151/181	141/181
ETH225	141/141	141/141	141/141	141/145
INRA023	203/203	203/203	203/203	185/211
ETH10	218/223	218/223	218/223	218/223

Fig. 1. A cloned Chikso calf bone at Day 291 after embryo transfer (female, 46kg). Tiger (striped) coat color is not yet revealed.

al., 1999; Zakhartchenko *et al.*, 1999; Kubota *et al.*, 2000). The reasons of these problems might be related to the incomplete reprogramming of a transferred donor nucleus resulted in abnormal gene expressions and functions (Smith and Murphy, 2004) including DNA methylation (Kang *et al.*, 2002), abnormal inactivation of X-chromosome (Xue *et al.*, 2002) and so on.

In this study, it was confirmed that NT-derived clone Chikso can produce after transfer of frozen-thawed NTs. The results, however, suggested that improvement of embryonic viability *in vivo* by the solution of abortion problem rather than technical problem of embryo transfer is very important to enhance the production efficiency of clone cattle. Moreover, it was needed that the control of parturition time and establishment of offspring management system to increase the postpartum survivability.

Prenatal characterization of SCNT embryos was relatively low pregnancy rate and high rate of early embryonic death. Dolly was resulted from only 8% of pregnancy rate and 3% of birth rate (Wilmut *et al.*, 1997). In mouse, implantation rate was high (more than 50%), but birth rate was only 2~3% (Wakayama *et al.*, 1998). In bovine SCNT studies, it was showed that 15 to 55% of pregnancy rates and 3 to 25% of birth rates (Cibelli *et al.*, 1998; Vignon *et al.*, 1998; Wells *et al.*, 1999), except one previous study (Kato *et al.*, 1998), in which 100% of pregnancy rate and 75~83% of birth rates were obtained after transfer of SCNT embryos. In the present study, pregnancy rate was about 35%, however, only one NT-derived calf was produced.

The characteristics of birth and postnatal period of SCNT clone animals were high birth weight, low survivability and high abnormality. In sheep SCNT, it was reported that gestation period varied from 132 to 161 days and birth weight also varied from 3.0 to 8.7 kg (Schnieke *et al.*, 1997), and 25 to 58% of offspring were stillbirth or early death after birth by abnormality (Schnieke *et al.*, 1997; Wilmut *et al.*, 1997). In bovine SCNT, large offspring syndrome was also one of the characteristics of clone fetus and offspring. Cibelli *et al.* (1998) reported that abortion at late gestation period was frequent in bovine SCNT due to the large body weight, edema of lung, large umbilical vein, and many other abnormalities. In this study, a clone calf was born with birth weight of 46 kg.

High proportion of early death after birth has been also reported in cattle NT (Cibelli *et al.*, 1998; Kato *et al.*, 1998; Wells *et al.*, 1999). A cause of postnatal death was an abnormally large pulmonary artery and umbilical vein (Cibelli *et al.*, 1998), acute hemorrhagic gastritis (Wells *et al.*, 1999), environmental factors (Kato *et al.*, 1998), umbilical cord infection and hematocelia (Shiga *et al.*, 1999; Vignon *et al.*, 1999), and so on. The clone calf produced in this study also infected in umbilical vessel.

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