Optimization of Electrofusion Condition for the Production of Korean Cattle Somatic Cell Nuclear Transfer Embryos

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ABSTRACT

This study was designed to determine the effect of electric field strength, duration and fusion buffer in fusion parameters on the rate of membrane fusion between the somatic cell and cytoplast for Korean cattle (HanWoo) somatic cell nuclear transfer (SCNT) procedure. Following electrofusion, effect of 5 or 10 μM Ca^{2+}-ionophore of activation treatment on subsequent development was also evaluated. Cell fusion rates were significantly increased from 23.1% at 20 V/mm to 59.7% at 26 V/mm and 52.9% at 27 V/mm ($p<0.05$). Due to higher cytoplasmic membrane rupture or cellular lysis, overall efficiency was decreased when the strength was increased to 30 V/mm (18.5%) and 40 V/mm (6.3%) and the fusion rate was also decreased when the strength was at 25 V/mm or below. The optimal duration of electric stimulation was significantly higher in 25 μs than 20 and 30 μs (18.5% versus 9.3% and 6.3%, respectively, $p<0.05$). Two nonelectrolyte fusion buffers, Zimmermann's (0.28 M sucrose) and 0.28 M mannitol solution for cell fusion, were used for donor cell and ooplast fusion and the fusion rate was significantly higher in Zimmermann's cell fusion buffer than in 0.28 M mannitol (91.1% versus 48.4%, respectively, $p<0.05$). The cleavage and blastocyst formation rates of SCNT bovine embryos activated by 5 μM Ca^{2+}-ionophore was significantly higher than the rates of the embryos activated with 10 μM of Ca^{2+}-ionophore (70.0% versus 42.9% and 22.5% versus 14.3%, respectively; $p<0.05$). This result is the reverse to that of parthenotes which shows significantly higher cleavage and blastocyst rates in 10 μM Ca^{2+}-ionophore than 5 μM counterpart (65.6% versus 40.3% and 19.5% versus 9.7%, respectively; $p<0.05$). In conclusion, SCNT couplet fusion by single pulse of 26 V/mm for 25 μs in Zimmermann's fusion buffer followed by artificial activation with 5 μM Ca^{2+}-ionophore are suggested as optimal fusion and activation methods in Korean cattle SCNT protocol.

(Key words : Electrofusion, Parthenogenetic activation, Somatic cell nuclear transfer, Bovine)

INTRODUCTION

After first transgenic cloned calf was born (Cibelli *et al.*, 1998), lots of reports were published relating to somatic cell nuclear transfer (SCNT) in cattle and this species has been one of the successful animal model for SCNT area. For the successful production of SCNT embryos, many experimental conditions including donor cell-to-ooplast fusion, oocyte activation and embryo culture should be precisely controlled and these conditions must be reproducible as well. In rodents, nuclear injection method is preferred to cell-to-cell fusion (Wakayama *et al.*, 1998; Roh, 2005). In some laboratories, nuclear injection was applied to bovine SCNT program using the isolated somatic cell nucleus as donor (Trounson *et al.*, 1998). However, in cattle, elecrofusion method is most popular for fusion between donor cells and enucleated oocytes.

Electric field-mediated fusion between mammalian cells is widely used in studying nuclear-cytoplasmic interactions in gametogenesis and early embryogenesis (Tarkowski, 1982) as well as in SCNT research. This induces lipid membrane fusion between a variety of cell types (Rickords and White, 1992). Although both inactivated Sendai virus and polyethylene glycol (PEG) had been used as fusion tools, electric fusion is generally recommended because it is simpler, less harmful and more repeatable method of fusing mammalian embryonic cells (Kubiak and Tarkowski, 1985). Electrofusion technique

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has two advantages of precisely measured repeatable parameters and short tem exposure to fusogenic agent ranged only μs. Electric field strength can be regulated by monitoring conductance of the fusion medium and adjusting the voltage between the electrodes to ensure maximum productivity (Rickords and White, 1992). Although many electrofusion parameters for SCNT in cattle were already reported by many research groups, the different electrofusion device and the different type of chamber can make different results for the efficient production of SCNT embryos regardless of optimal conditions suggested by the authors. A low-conductive, nonelectrolyte fusion solution (for example, mannitol) has been recommended as electrofusion buffer to prevent conductive heat buildup, which can damage early embryonic cells (Robl and Stice, 1989) although electrolyte buffer can be used for the fusion of two-cell embryos (Tsunoda *et al.*, 1987).

The present study was designed to optimize the condition of electrofusion parameters (direct currency voltage and pulse duration) and to choose an appropriate fusion buffer (Zimmermann's cell fusion buffer versus 0.28 M mannitol, which are most commonly used for cell fusion of cell couplets) for the efficient production of SNCT embryos in Korean cattle, HanWoo. Prior to perform the experiments above, activation condition was also tested using the parthenogenetic bovine embryos to optimize the productivity of SCNT embryos.

MATERIALS AND METHODS

Chemicals

All inorganic and organic compounds were purchased from Sigma-Aldrich Korea unless indicated otherwise.

Oocyte Recovery and *In Vitro* Maturation (IVM)

Bovine ovaries were collected at a local slaughterhouse and transported to the laboratory within 2~3 h in saline at 25~35℃. Cumulus-oocyte complexes (COCs) were recovered by aspiration of 3 to 8 mm follicles using an 18 gauge hypodermic needle attached to a 10 ml disposable syringe. After washing three times in IVM medium, COCs that were enclosed by more than three layers of compact cumulus cells and an evenly granulated ooplasm were selected for IVM. Selected COCs were cultured in 4-well culture dishes (Nunc, Denmark) containing 500 μl of IVM medium under warmed and gas-equilibrated mineral oil for 20~22 h at 38.5℃, 5% CO_2. The IVM medium for oocytes is composed of tissue culture medium 199 with Earle's salts and L-glutamine (TCM199, Invitrogen, USA) supplemented with 10% FBS (Invitrogen), 10 μg/ml FSH-P (Follitropin-V, Veterpharm, UK), 0.2 mM sodium pyruvate, 1 μg/ml estradiol-17β and 10 ng/ml EGF.

Parthenogenetic Activation and IVC

Oocyte activation was performed after IVM. The oocytes were incubated in CR2 medium (Rosenkrans and First, 1994) supplemented with 0.1% PVA and either 5 or 10 μM Ca-ionophore for 5 min. Then, CR2 medium supplemented with 2 mM 6-dimethylaminopurine (6-DMAP) for 3 h. After the treatment, the activated oocytes were transferred to IVC medium consisting of CR2 with 0.3% fatty acid-free BSA (ff-BSA) and 1% v/v insulin, transferrin and selenium complex (ITS) for 3 days and then transferred to CR2 medium with 0.15 % ff-BSA, ITS and 0.15 % FBS for 5 days at 39℃ in 5% CO_2, 5% O_2 and 90% N_2. The culture drops were covered by mineral oil and 10 to 15 embryos were placed in each drop.

Preparation of Donor Cells

Bovine ear skin (Korean cattle) tissue was cut into small pieces with scissors. Cells were suspended with Dulbrcco's modified Eagle medium (DMEM; Invitrogen) containing 10% FBS, and the suspension was centrifuged at 1,200 rpm for 5 min. The cell pellet was resuspended and cultured in DMEM supplemented with 10% FBS and antibiotics. For donor cells to be used in SCNT, bovine ear skin cells were cultured until they reached confluence. Before SCNT, cells were treated with 0.05% trypsin for single-cell isolation. After 4~7 passages, these cells were used as donor cells for SCNT.

Enucleation and Nuclear Transfer

Matured oocytes were enucleated in Hepes-buffered TCM199 (hTCM199) supplemented with 20% FBS. 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 the adjacent cytoplasm presumably containing the metaphase II chromosomes were extruded by squeezing with the needle. The enucleated oocytes were placed and incubated in hTCM199 (with 10 % FBS) until SCNT occurred. A single cell with and intact membrane was selected and transferred by micropipette into the perivitelline space of the enucleated oocyte through the slit made during enucleation.

Electrofusion and Activation

Karyoplast-cytoplast complexes were incubated either in Zimmermann's cell fusion medium (consisting of 0.28 M sucrose, 0.5 mM $Mg(C_2H_3O_2)_2 \cdot 4H_2O$, 0.1 mM $Ca(C_2H_3O_2)_2$, 1.0 mM K_2HPO_4, 0.1 mM glutathione) or in 0.28 M mannitol with 0.1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ for equilibration and transferred into a cell fusion chamber. Then the couplets were manually ori-

ented by fine electrical rods under light microscopy. Cell fusion was accomplished with a single DC pulse of 10~40 V/mm for 20~30 μs upon the designs of the experiments. Electric stimulation was delivered by Embryonic Cell Fusion System™ (Fujihira Industry Co., Ltd., Tokyo, Japan). Following electric stimulation, donor cell-cytoplast complexes were incubated in hTCM-199. After 30 min of electric stimulation, fusion was confirmed under a stereomicroscope. Activation and IVC of reconstructed SCNT oocytes was the same as for the parthenogenetic embryos.

Experimental Designs

In experiment 1, the effect of the concentration of Ca^{2+}-ionophore during the activation on *in vitro* development of bovine parthenotes was evaluated. Parthenogenetic activation was performed with 5 or 10 μM Ca^{2+}-ionophore for 5 min and was followed by 2.5 mM 6-DMAP for 3 h. After activation treatment, the pathenotes were transferred to CR2 medium and culture for 7 days. Efficacy of pathenogenetic activation was assessed by cleavage and blastocyst formation rates.

In experiment 2, the effects of electric pulse strength and duration on the fusion of SCNT couplets were evaluated. First, the couplets were fused with a single DC pulse of 10, 15, 18, 19, 20, 25, 26, 27, 30 or 40 V/mm for 20 μs to identify optimal pulse strength. Second, the couplets were fused with a single DC pulse of 26 V/mm for 20, 25 or 30 μs to identify optimal pulse duration.

In experiment 3, two kinds of nonelectrolyte fusion buffers, Zimmermann's fusion buffer and 0.3 M mannitol, were compared to select appropriate fusion buffer for SCNT program. Pulse strength and duration was fixed to 26 V/mm for 25 μs in each group.

In experiment 4, the effect of the concentration of Ca^{2+}-ionophore during the activation on *in vitro* development of SCNT bovine embryos was evaluated. The experiment was the same as experiment 1 except using SCNT couplet for activation, not parthenotes.

Statistical Analysis

Experiments were repeated more than three times, and the proportion of cleavage and the blastocyst formation were evaluated by Student's *t*-test. Difference at $p<0.05$ was considered significant.

RESULTS

Effect of the Concentration of Ca^{2+}-ionophore during the Activation on *In Vitro* Development of Bovine Parthenotes

The cleavage and blastocyst formation rates of parthenogenetic oocytes activated by 10 μM Ca^{2+}-ionophore was significantly higher than the rates of the oocytes activated with 5 μM Ca^{2+}-ionophore (65.6% versus 40.3 % and 19.5% versus 9.7%, respectively; $p<0.05$, Table 1).

Table 1. Effect of the concentration of Ca^{2+}-ionophore during the activation on the blastocyst development of bovine parthenotes *in vitro*

Activation condition*	No. of oocytes	No. (%) of parthenotes developed to	
		Cleaved	Blastocysts
5 μM Ca^{2+}-ionophore	62	25 (40.3) [a]	6 (9.7) [a]
10 μM Ca^{2+}-ionophore	87	57 (65.5) [b]	17 (19.5) [b]

* 5 μM Ca^{2+}-ionophore : 5 μM Ca^{2+}-ionophore for 5 min + 2.5 mM 6-DMAP for 3 h.
5 μM Ca^{2+}-ionophore : 10 μM Ca^{2+}-ionophore for 5 min + 2.5 mM 6-DMAP for 3 h.
[a,b] Values in the same column with different letters superscripts differ significantly ($p<0.05$).

Effects of Electric Pulse Strength and Duration on the Fusion of SCNT Couplets

Cell fusion rates were significantly increased from

Table 2. Fusion rates of bovine SCNT couplets by the application of single electric pulse with different voltages ranged from 10 to 40 V

Electric pulse strength*	No. of oocytes	No. (%) of reconstructed oocytes	
		Fused	Not fused
10 V	36	0 (0) [a]	36
15 V	36	0 (0) [a]	30
18 V	54	10 (18.5) [c]	44
19 V	18	2 (11.1) [c]	16
20 V	52	12 (23.1) [c]	40
25 V	119	46 (38.7) [d]	73
26 V	139	83 (59.7) [d]	53
27 V	68	36 (52.9) [d]	32
30 V	27	5 (18.5) [c]	18
40 V	16	1 (6.3) [b]	14

* Direct currency per mm, pulse duration was fixed to 20 μs.
[a-d] Values in the same column with different letters superscripts differ significantly ($p<0.05$).

Table 3. Fusion rates of bovine SCNT couplets by the application of single electric pulse with different durations ranged from 20 to 30 μs

Duration of electric pulse*	No. of oocytes	No. (%) of reconstructed oocytes	
		Fused (%)	Not fused
20 μs	43	4 (9.3) [a]	39
25 μs	27	5 (18.5) [b]	22
30 μs	16	1 (6.3) [a]	15

* Pulse strength of direct currency was fixed to 26 V/mm, single pulse applied.
[a,b] Values in the same column with different letters superscripts differ significantly ($p<0.05$).

23.1% at 20 V/mm to 59.7% at 26 V/mm and 52.9% at 27 V/mm ($p<0.05$, Table 2). Due to higher cytoplasmic membrane rupture or cellular lysis, overall efficiency was decreased when the strength was increased to 30 V/mm (18.5%) and 40 V/mm (6.3%) and the fusion rate was also decreased when the strength was at 25 V/mm or below. The optimal duration of electric stimulation was significantly higher in 25 μs than 20 and 30 μs (18.5% versus 9.3% and 6.3%, respectively; $p<0.05$, Table 3).

Comparison of Two Nonelectrolyte Fusion Buffers for Cell Fusion

Cell fusion rate was significantly higher in Zimmermann's cell fusion buffer than in mannitol buffer (91.1 % versus 48.4%, respectively; $p<0.05$).

Effect of the Concentration of Ca^{2+}-ionophore during the Activation on *In Vitro* Development of SCNT Bovine Embryos

The cleavage and blastocyst formation rates of SCNT bovine embryos activated by 5 μM Ca^{2+}-ionophore was significantly higher than the rates of the embryos activated with 10 μM of Ca^{2+}-ionophore (70.0% versus 42.9 % and 22.5% versus 14.3%, respectively; $p<0.05$, Table 5). This result is the reverse to that of parthenotes.

Table 4. Comparison of nonelectrolyte fusion buffers for the fusion of bovine SCNT couplets

Fusion buffer*	No. of oocytes	No. (%) of reconstructed oocytes	
		Fused (%)	Not fused
Mannitol	128	61 (48.4) [a]	67
Zimmermann	45	41 (91.1) [b]	4

* Mannitol: 0.1 mM $CaCl_2$ + 0.5 mM $MgCl_2$ + 0.28 M mannitol. Zimmermann: 0.28 M sucrose + 0.5 mM $Mg(C_2H_3O_2)_2 \cdot 4H_2O$ + 0.1 mM $Ca(C_2H_3O_2)_2$ + 1.0 mM K_2HPO_4 + 0.1 mM glutathione.
[a,b] Values in the same column with different letters superscripts differ significantly ($p<0.05$).

Table 5. Effect of the concentration of Ca^{2+}-ionophore during the activation on the blastocyst development of bovine SCNT embryos *in vitro*

Activation condition*	No. fused	No. (%) of reconstructed oocytes	
		Cleaved	Blastocysts
5 μM-Ca	40	28 (70.0) [a]	9 (22.5) [a]
10 μM-Ca	28	12 (42.9) [b]	4 (14.3) [b]

* 5 μM Ca: 5 μM Ca^{2+}-ionophore for 5 min + 2.5 mM 6-DMAP for 3 h.
10 μM Ca: 10 μM Ca^{2+}-ionophore for 5 min + 2.5 mM 6-DMAP for 3 h.
[a,b] Values in the same column with different letters superscripts differ significantly ($p<0.05$).

DISCUSSION

Factors affecting the success of animal cloning include activation protocols (Shin *et al.*, 2001; Choi *et al.*, 2004) and fusion conditions (Karja *et al.*, 2006; Park *et al.*, 2011). In the present study, we compared the effect of different fusion conditions to determine the effective bovine cloning procedures. The data presented here demonstrated that artificial activation with 5 μM Ca^{2+}-ionophore following electrofusion with single DC pulse of 26 V/mm for 25 μs in Zimmermann's fusion buffer achieved most efficient outcome in Korean cattle SCNT program.

A wide range of chemical activators have been proposed in attempt to mimic natural biochemical and morphological actions of sperm during fertilization. Intracellular calcium oscillations can influence the latter stage of embryo development by calcium concentration change. Ozil and Huneau (2001) reported that changes in calcium inflex during oocyte activation affect the post-implantation embryo development in rabbits, due to interference on epigenetic reprogramming of the zygote genome. Theses epigenetic abnormalities are transmitted though blastomere divisions and result in changes of gene expression of antiapoptotic and proapoptotic genes in preimplantation embryos. Wrenzycki *et al.* (2001) demonstrated that modifications of activation protocol can also alter the expression pattern of developmentally important genes in SCNT-derived embryos. Although no single activation agent available that can induce physiological Ca^{2+} oscillations capable of supporting development (Ware *et al.*, 1989; Knott *et al.*, 2002), contemporary protocols for activation of reconstructed bovine oocytes commonly use a calcium mobilizing compound like calcium ionophore, ethanol and strontium as a primary treatment using either cycloheximide (Zakhartchenko *et al.*, 1999) or 6-DMAP (Wells *et al.*, 1999). However, optimal condition of Ca^{2+}-ionophore need to

be evaluated as the condition suggested by various research groups are different and it may be affected by the equipments and technicians etc. In our laboratory, as shown in Table 1 and 5, treatment of 10 μM Ca^{2+}-ionophore was optimal for pathenote whereas 5 μM for SCNT embryo. The different outcome between parathenote and SCNT embryo can be explained that electrical stimulation for cell fusion before activation in SCNT protocol may trigger an influx of extracellular Ca^{2+} immediately after the stimulation (Sun *et al.*, 1992) and enables to activate fused SCNT couplet with lower chemical stimulation.

Electrofusion occurs as a result of membrane breakdown and pore formation between cell to cell and it has been clearly demonstrated that the electrical breakdown of plasma membrane is dependent on pulse strength and duration (Zimmermann and Vienken, 1982). The efficiency of fusion increases with the rise in the electric field strength and duration until these values reach a critical level (Ozil and Modlinski, 1986). However, higher levels of intracellular Ca^{2+} caused by inappropriate of pulse duration and electric field strength have a detrimental effect on the cell function or structures (Izant, 1983). The success of electric fusion requires precise alignment of the membranes to be fused perpendicular to the electric current, close contact between these membranes, and delicately timed specific duration and strength of the applied electric field. In the present study we found that optimal fusion condition is 26 or 27 V/mm for 25 μs. Since the strength over 30 V/mm shows cellular damages including membrane rupture, this may be the upper limit for application to SCNT program. On the other hand, the condition under 25 V/mm showed lower fusion rate and this may be from incomplete membrane breakdown and/or pore formation between donor cell and ooplasm. Duration of electrical pulse results in an alternation of the level of intracellular calcium. Insufficient or excessive Ca^{2+} stimulation related to pulse duration impairs blastocyst development (Fissore *et al.*, 1992) and the optimal duration we found here is 25 μs when electric strength is fixed to 26 V/mm.

For electrofusion of mammalian cells, fusion buffer should be of low conductance to promote the establishment of cellular dielectric potentials within cells, which facilitates intercellular contact and reduces the current-generated temperature increase in medium immediately following electric pulse. Sugars of isosmotic concentration in fusion buffer such as glucose, sucrose, sorbitol or mannitol maintain osmotic pressure and decrease the conductivity of pulse in the buffer (Zimmermann and Vienken, 1982). It is important to consider the ionic composition of the cytosol to prevent detrimental ion concentration gradients that may occur after electric pulse induced membrane pore formation (Zimmermann *et al.*, 1984). The present study demonstrated that Zimmermann's fusion buffer containing sucrose as its sugar component showed higher fusion rates than mannitol solution. Osmotic pressures of Zimmermann's and mannitol were identical as 280mOsm and both solutions also include Ca^{2+} and Mg^{2+}, important factors for fusion efficiency. Although both fusion buffers are commonly used for SCNT program in cattle, in our laboratory condition, Zimmermann's buffer is more suitable for fusion in Korean cattle SCNT program. However, no clear explanation can be claimed here.

In conclusion, SCNT couplet fusion by single pulse of 26 V/mm for 25 μs in Zimmermann's fusion buffer followed by artificial activation with 5 μM Ca^{2+}-ionophore are suggested as optimal fusion and activation methods in HanWoo SCNT protocol.

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