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In Vitro Development of Bovine Nuclear Transfer Embryos Reconstructed with Fetal Fibroblasts

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ABSTRACT

The present study was to examine effects of various electrical stimulus treatments used for electrofusion on the preimplantation development of bovine nuclear transfer (NT) embryos with fetal fibroblast cells. Fetal fibroblast cells were isolated from one fetus at day 45 of gestation in Holstein cow, and passaged 3 to 4 times before being transferred into enucleated oocytes. Single fibroblast cells were individually placed into the perivitelline space of enucleated oocytes by using a micromanipulator. At first, the fusion and developmental rates of reconstructed oocytes were compared between different electric stimulation conditions. When fusion of the reconstructed oocyte was induced by different electric pulse periods (15, 30 and 45 μsec) at a DC pulse of 1.8 kV/cm, 15 (45.5%, 120/264) or 30 μsec group (43.9%, 106/241) showed a higher fusion rate than 45 μsec group (23.2%, 58/250, $P<0.05$). However, no difference was detected in the development rate of the fused oocytes to blastocysts between groups. Next experiment was to examine the effects of different electrical field strengths (1.5, 1.8 and 2.1 kV/cm) for 15 μsec at electrofusion on *in vitro* development of the NT embryos. As results, there was no difference in the fusion and developmental rates of the NT embryos between electrical strength ($P>0.05$). Finally, developmental competence of bovine NT embryos with somatic cells was compared with IVF-derived embryos. Of enucleated oocytes fused with fibroblast cells, 27.4% (75/274) developed to the blastocyst stage, which is similar to that (24.5%, 58/237) of IVF-derived embryos. However, mean nuclei number of NT blastocysts was smaller than that of IVF-derived blastocysts.

Thus, we have established an optimal condition (1.8 kV/cm, 15 μsec) for electric fusion of bovine NT oocytes with somatic cells. The present study indicates that bovine reconstructed embryos with somatic cells normally develop to blastocyst stage *in vitro*, although having smaller nuclei numbers of blastocysts as compared to IVF-derived embryos.

(Key words : Electrofusion, Nuclear transfer, Fetal fibroblasts, Bovine)

I. INTRODUCTION

Successful clone cattle have been achieved from various somatic cells (Cibelli et al., 1998; Kato et

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al., 1998; Wells et al., 1999; Zakhartchenko et al., 1999abc). Fetal fibroblast cells have been used to produce clone livestock such as sheep (Schnieke et al., 1997), goat (Baguisi et al., 1999), pig (Onishi et al., 2000) and cattle (Cibelli et al., 1998) because of their potential for many cell divisions before senescence in culture.

The fusion of a donor nuclei and an enucleated oocyte is necessarily needed to generate developing NT embryos. Electric stimulus method has been generally used for fusion of cultured somatic cells and enucleated oocytes in various species (Campbell et al., 1996; Wells et al., 1997; Hill et al., 2000; Miyoshi et al., 2000). Here, combined conditions of the electric field strength and pulse duration used for fusion may be dependent on distance between two electrodes and species. The electrical variation may be one of factors which affect the development of NT embryos. However, little information is known about detailed electric conditions for fusion in the somatic cell nuclear transfer.

In earlier studies, it has been accepted that the donor nucleus for nuclear transfer must be in G1 or G0 (Prather et al., 1992; Campbell et al., 1994), although it is not clear if G1 or G0 is more appropriate. However, which stage of cells in the cell cycle results in the development of NT embryos after nuclear transfer is still controversial. Wilmut et al. (1997) have suggested that the donor cells for nuclear transfer must be in G0 of the cell cycle, a quiescent phase. Nuclei of starved fetal fibroblasts in bovine showed a higher development of reconstructed embryos to the blastocyst stage than non-starved fibroblasts (Zakhartchenko et al., 1999c; Hill et al., 2000), although serum starvation of the adult donor cells did not improve preimplantation development of NT embryos (Hill et al., 2000). Alternatively, cycling cultured cells from a fetus could be successfully used for nuclear transfer in cattle (Cibelli et al., 1998). Vignon et al. (1999) reported that there was no difference in development of NT embryos between quiescent and proliferative fibroblasts. In this study we have used fetal fibroblast cells without serum starvation to test the development potential of reconstructed embryos after nuclear transfer.

In the present study the effects of electrical pulse duration and field strengths on development of bovine NT embryos with somatic cells were investigated. In addition, developmental competence of NT embryos was compared with that of IVF-derived embryos.

Ⅱ. MATERIALS AND METHODS

1. *In Vitro* Maturation (IVM)

Unless otherwise mentioned, all chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Bovine ovaries were collected at a slaughterhouse and transported to the laboratory and then washed in 0.9% saline at 25~30℃. Cumulus oocyte complexes (COCs) were aspirated through an 18-gauge needle into a disposable 10 ml syringe from follicles 2 to 8 mm in diameter. COCs were selected in TL-HEPES medium (Prather et al., 1995), washed 3 times with maturation medium and randomly allocated in groups of 30~35 in each well of a 4-well culture plate (Nunc, Roskilde, Denmark) containing 500 μl of TCM199 (GibcoBRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; GibcoBRL), 10 μg/ml p-FSH (Folltropin-V, Vetrepharm, London, UK), 0.6 mM cysteine, 0.2 mM Na-pyruvate, 25 μg/ml gentamycin and 1 μg/ml estradiol-17β. Oocytes were cultured at 38.5℃ in a humidified atmosphere of 5% CO_2 in air.

2. *In Vitro* Fertilization (IVF)

At 22 to 24 h of culture, oocytes were rinsed twice with TALP (Bavister and Yanagimachi, 1977) and placed in 50 μl of fertilization medium, which consisted of modified Tyrode-Lactate medium (Fert-TALP; Bavister and Yanagimachi, 1977). Frozen semen from two Holstein bulls was thawed at 37℃ in water for 30 sec and layered on a 45 to 90% Percoll gradient in a 15 ml centrifuge tube. The Percoll gradient was composed of 2 ml of 90% Percoll overlaid with 2 ml of 45% Percoll, both of which were prepared with Sperm TALP (SP-TALP; Parrish et al., 1988). After 20 min of centrifugation at 300×g, the top layers were removed and the sperm pellet was suspended in SP-TALP. After 10 min of centrifugation at 150×g, the sperm pellet was resuspended by the same method. The sperm suspension was introduced into a 50 μl drop of fertilization medium containing 10 oocytes to make the final sperm concentration ($1\sim2\times10^6$/ml). When sperm were added to the fertilization drops, 2 μg/ml heparin, 20 μM penicillamine, 10 μM hypotaurine and 1 μM epinephrine (PHE) were also added. Spermatozoa and oocytes were co-incubated at 38.5℃ in 5% CO_2 in air for 20~22 h.

3. Preparation of Fetal Fibroblast Cells

Fetal fibroblast cells were isolated from fetus at day 45 of gestation in the Holstein cow. The sex of the fetus was male as determined by PCR. Primary fetal fibroblast cells were prepared as described previously (Zakhartchenko et al., 1999c). Briefly, the head of fetus was removed using iris scissors and soft tissues such as liver and intestine were also discarded by scooping out with two watch-makers forceps. After twice washing with PBS, the carcass was minced with a surgical blade on 100 mm culture dish. The minced tissues were incubated in 10 ml of 0.05% (w/v) trypsin/0.53 mM EDTA solution in an incubator at 39℃ for 30 min. Trypsin activity was inhibited by adding an equal volume of cell culture medium supplemented with 10% FBS. The cell culture medium was composed of Dulbecco's modified Eagle's medium (DMEM; GibcoBRL), 10% FBS, 1,000 units of penicillin (GibcoBRL) and 1 mg/ml of streptomycin (GibcoBRL). After vigorous pipetting, the supernatant was centrifuged at 150×g for 5 min. The cells were suspended, adjusted to a final concentration of 2×10^6 cells/ml and then cultured in 10 ml of the culture medium at 39℃, 5% CO_2 in air into 175 cm^2 tissue culture flask (Nunc) until confluent. The fetal fibroblast cells were passaged 3 to 4 times before use as donor nuclei.

4. Oocyte Enucleation

After 22 h of culture, mature oocytes were freed of cumulus cells by pipetting in 500 μl of TL-HEPES medium supplemented with 0.1% hyaluronidase. Before enucleation, the zona pellucida of a denuded oocyte was partially dissected with a fine glass needle. The method used for cutting of zona was described in a previous report (Tsunoda et al., 1986). Enucleation of oocytes was performed by using a Leitz inverted microscope fitted with Normarski optics and a Leitz micromanipulation system (Leitz, Ernst Leitz Wetzlar GmbH, Germany). Briefly, oocytes were placed into a 20 μl microdrop of TL-HEPES containing 7.5 μg/ml cytochalasin B under mineral oil. The first polar body and the adjacent cytoplasm were aspirated through a micropipette with an inner diameter of 20 μm together. After enucleation, the oocytes were stained with 5 μM bisbenzimide 33342 for 5 min, and then individually scrutinized for the presence of maternal nuclear materials under an epifluorescent microscopy. Oocytes showing DNA materials were excluded in subsequent experiments.

5. Nuclear Transfer, Activation and Embryo Culture

Fibroblasts at confluence after 2 d of culture were trypsinized and washed twice with PBS. A single fibroblast was placed into perivitelline space of an enucleated oocyte. The reconstructed oocytes were equilibrated in 0.3 M Mannitol cell fusion medium for 1 min, and then transferred to a fusion chamber consisting of two electrodes 1 mm apart, overlaid with fusion medium. The reconstructed eggs were exposed to a single electric DC pulse of 1.8 kV/cm for 15 μsec (BTX Electro Cell Manipulator 2001, Inc., San Diego, CA) in order to initiate their fusion (Cibelli et al., 1998).

At 4 h after electrofusion, the fused eggs were chemically treated with 5 μM ionomycin for 5 min followed by treatment with 2.5 mM 6-DMAP (6-dimethyl-aminopurine) in CR_{1aa} supplemented with 10% FBS for 3.5 h. After activation, the embryos were washed 5 times and then cultured in CR1aa supplemented with 3 mg/ml BSA (Rosenkrans and First, 1991). After culture of 3 d, cleaved embryos were further co-cultured in each well of 4-well culture plate containing 750 $\mu\ell$ CR_{1aa} supplemented with 10% FBS on mouse embryonic fibroblasts (MEFs) monolayer for 4 d (Park et al., 2000). IVF-derived bovine eggs were also cultured as described above.

6. Nuclei Numbers of Blastocysts

Blastocysts that developed from NT or IVF -derived embryos were fixed in 1% formalin solution for 10 min at room temperature and then placed on slides into a drop of mounting medium which consisted of glycerol : PBS (3:1) containing 2.5 mg /ml sodium azide and 2.5 μg/ml Hoechst 33342. A cover slip was placed on the top of the medium and the edge was sealed with fingernail polish. The nuclei number of blastocysts were counted under an Olympus fluorescence microscope (Olympus, Japan).

7. Chromosome Analysis

Bovine NT blastocysts were cultured in CR_{1aa} medium containing colcemid (0.05 μg/ml) overnight. A single blastocyst was placed in hypotonic solution (0.56% KCl) for 5 minutes, directly mounted on a glass slide and then fixed with fixative (methanol : glacial acetic acid = 3 : 1). After air-drying, the slides were stained with 5% (v/v) Giemsa for 15 minutes and washed with distilled water. Metaphase spreads from the embryos were observed on a microscope under 1000 magnification.

8. Experimental Designs

1) Experiment 1

It was examined whether different fusion treatments affect *in vitro* development of NT bovine embryos to blastocyst stage. At electrofusion, approximately one-third of NT embryos were simultaneously allocated to 3 electrical pulse period groups (15, 30 and 45 μsec pulse at 1.8 kV/cm DC) and 3 electrical strength groups (1.5, 1.8 and 2.1 kV/cm DC for 15 μsec), respectively. After electrofusion, the reconstructed embryos were treated with A23187 (5 μM for 5 min) and then incubated in CR_{1aa} medium supplemented with 2 mM 6-DMAP for 4 h. After activation treatments, the reconstructed embryos from each group were washed 4 times and cultured in CR_{1aa} medium containing 3 mg/ml BSA.

2) Experiment 2

Developmental potential of NT bovine embryos with fibroblast cells was compared to IVF-derived embryos. At 2 h after electrofusion, NT embryos were treated with 5 μM A23187 for 5 min and then incubated in CR1aa medium supplemented with 2.5 mM 6-DMAP for 4 h. Both IVF-derived and NT embryos were cultured in CR_{1aa} medium containing 3 mg/ml BSA.

In experiments 1 and 2, the embryos were cul-

tured in 50 $\mu\ell$ drops of CR_{1aa} medium supplemented with 3 mg/ml BSA at 38.5℃, 5% CO_2 in air. After culture of 3 d, cleaved embryos were further co-cultured with MEF monolayer at 38.5℃, 5% CO_2 in air for 3 d. After 7 d of culture, blastocyst formation was observed and then nuclei number of blastocysts were counted on a fluorescent microscope following Hoechst 33342 (2.5 μg/ml) staining.

9. Statistical Analyses

Statistical analyses of data from at least 4 replicates were carried out by Student's t-test. Probability of $P<0.05$ was considered to be statistically significant.

Ⅲ. RESULTS

1. Effects of Electric Pulse Durations and Field Strengths on the Fusion and Development Rates of NT Oocytes

It was first examined whether different pulse durations at 1.8 kV/cm electric pulse in the fusion of NT embryos with somatic cells affect *in vitro* development of NT bovine embryos to blastocyst stage. As shown in Table 1, the NT oocytes fused at 45 μsec had a lower fusion rate (23.2%, 58/250) than 15 (45.5%, 120/264) and 30 μsec group (43.9%, 106/241) ($P<0.05$). This difference was due to a high proportion of lysed oocytes (59.6%, 149/250) as compared to 15 (6.4%, 17/264) and 30 μsec groups (23.7%, 57/241). However, *in vitro* development rate of the fused embryos was not different between groups. In next experiment, we examined the effect of electric field strengths on *in vitro* development of NT oocytes under the condition of 15 μsec pulse duration (Table 2). The fusion rates of NT oocytes at 1.5, 1.8 and 2.1 kV/cm pulses were 44.0% (77/175), 45.9% (78/170) and 36.9% (66/179), respectively. No difference was also observed in the blastocyst formation between groups. Thus, the results demonstrate that a single electric DC pulse of 1.8 kV/cm for 15 μsec can support a higher fusion rate of bovine NT oocytes with fetal fibroblast cells.

2. *In Vitro* Development of NT Embryos

Developmental competence of bovine oocytes reconstructed with somatic cells was compared to IVF-derived embryos (Table 3). The cleavage rate of NT embryos (72.3%, 198/274) was slightly lower than that of IVF embryos (82.7%, 196/237), although there was not significant difference ($P>0.05$). Developmental rate (27.4%, 75/274) to blastocyst stage of NT embryos was similar to that (24.5%, 58/237) of IVF embryos. Most NT blastocysts appeared to

Table 1. Effect of electric pulse duration on fusion and development rates of reconstructed bovine oocytes with fetal fibroblast cells

Pulse duration	No. (%) of embryos		No. (%)* of embryos cleaved	No. (%)** of blastocysts
	Examined	Fused		
15 μsec	264	120 (45.5)[a]	90 (75.0)	33 (27.5)
30 μsec	241	106 (43.9)[a]	75 (70.8)	23 (21.7)
45 μsec	250	58 (23.2)[b]	43 (74.1)	15 (25.9)

* No. of embryos cleaved/No. of oocytes fused × 100.

** No. of blastocysts/No. of oocytes fused × 100.

[ab] Different superscripts within columns denote significant differences ($P<0.05$).

Table 2. Effect of electrical field strength on fusion and development rates of reconstructed bovine oocytes with fetal fibroblast cells

Voltages	No. (%) of embryos		No. (%)* of embryos cleaved	No. (%)** of blastocysts
	Examined	Fused		
1.5 kV/cm	175	77 (44.0)	61 (79.2)	20 (25.9)
1.8 kV/cm	170	78 (45.9)	59 (75.6)	22 (28.2)
2.1 kV/cm	179	66 (36.9)	51 (77.3)	18 (27.3)

* No. of embryos cleaved/No. of oocytes fused × 100.

** No. of blastocysts/No. of oocytes fused × 100.

Table 3. *In vitro* development of bovine NT embryos reconstructed with fetal fibroblast cells for 7 d

Group	No. of embryos examined	No. (%) of embryos cleaved	No. (%) of blastocysts
IVF	237	196 (82.7)	58 (24.5)
NT	274	198 (72.3)	75 (27.4)

Table 4. Nuclei number of bovine blastocysts that developed from NT or IVF-derived embryos

Group	Blastocyst stage	No. of embryos examined	Nuclei no. (Mean±SD)
IVF	Early	14	76.7 ± 12.2^{a}
	Expanded	11	131.4 ± 18.1^{b}
NT	Early	11	50.9 ± 9.1^{c}
	Expanded	12	103.8 ± 23.5^{d}

[abcd] Values with different superscripts are different significantly ($P<0.05$ for same embryo stage, $P<0.01$ for different embryo stage).

be hatched after 7 d of culture (data not shown). Thus, our data show that nuclei of fetal fibroblast cells transferred into the enucleated oocytes support *in vitro* development of NT embryos to blastocyst stage like IVF-derived embryos.

3. Nuclei Numbers of NT Blastocysts

Nuclei numbers of NT blastocysts were compared to IVF-derived blastocysts to evaluate putatively the embryonic developmental potential (Table 4). After Hoechst 33342 (2.5 μg/ml) staining, nuclei of the embryos were counted by using a fluorescent microscope (Fig. 1). The nuclei number of NT blastocysts ranged from 37 to 64 in early blastocyst stage and from 62 to 137 in expanded blastocyst stage, while IVF-derived blastocysts ranged from 55 to 91 in early stage and from 100 to 160 in expanded stage. As shown in Table 4, mean nuclei numbers (50.9 ± 9.1 and 103.8 ± 23.5, respectively) of NT blastocysts in early and expanded stages were significantly smaller than those (76.7 ± 12.2 and 131.4 ± 18.1, respectively) of IVF-derived blastocysts ($P<0.05$). In another experiment, it was observed whether NT blastocysts derived from somatic cells have normal karyotypes. Of 20 blastocysts examined by cytological analysis, 14 embryos having 25 countable metaphase spreads showed normal diploid chromosomes (60XY). Consequently, the present study suggests that the reconstructed oocytes with somatic cells have *in vitro* developmental potential to normal blastocysts, although showing a smaller nuclei numbers than IVF-derived embryos.

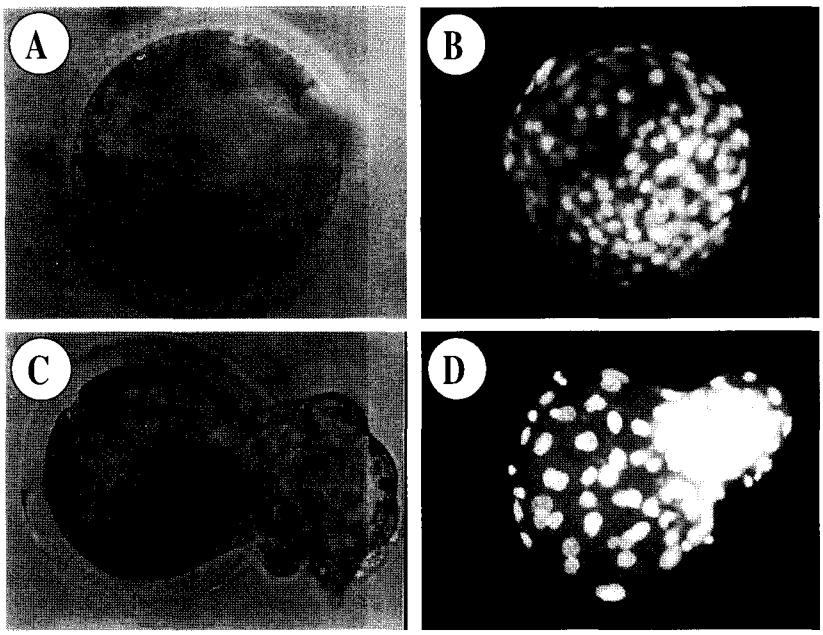

Fig. 1. Bovine blastocysts that developed from IVF-derived (A) and NT embryos (C). Hoechst 33342 stained IVF-derived (B) and NT blastocysts (D).

Ⅳ. DISCUSSION

In this study, we have tried to establish optimal electric conditions for fusion of a recipient cytoplast and a donor cell. in the somatic cell nuclear transfer. In somatic cell nuclear transfer, electrofusion has been generally carried out to introduce donor nuclei into enucleated oocytes in various species (Campbell et al., 1996; Wells et al., 1997; Hill et al., 2000; Miyoshi et al., 2000). Here, electrical pulse duration and field strength may be important factors to enhance the efficiency of nuclear transfer. A variety of electrofusion methods have been reported. Many laboratories have employed double pulses of electricity for the fusion of NT oocytes (Kato et al., 1998; Wells et al., 1999; Hill et al., 2000). However, when the two pulse system applied to this study, all reconstructed oocytes were lysed within 1h after electrofusion (data not shown). This difference may be due to a different kind of fusion chamber although there is uncertain of its validation. Some laboratories have used a chamber consisting of parallel wires (500 μm apart) for fusion (Wells et al., 1999; Zakhartchenko et al., 1999c). In this study we have employed a single electric pulse (1.8 kV/cm for 15 μsec) for fusion of a enucleated oocyte and a donor cell by using a chamber consisting of parallel wires with a separation of 1 mm. The electric conditions (1.8 kV/cm for 15 μsec) used for fusion of NT embryos in our trials are same as previously reported by Cibelli et al. (1998).

Our results indicate that the actively dividing fibroblast cells can support development to blastocyst stage of reconstructed bovine embryos after nuclear transfer. The fact that serum starvation is not essential for the production of successful clone cattle has been evidenced by other reports (Cibelli et al., 1998; Zakhartchenko et al., 1999c; Kubota et

al., 2000). Vignon et al. (1999) also demonstrated that no difference was observed in the *in vitro* development of NT embryos between quiescent and proliferative fibroblasts. The *in vitro* developmental rate (27.4%) of bovine NT embryos obtained in this study was high as compared with that (12%) of another paper (Cibelli et al., 1998). However, this result was slightly lower than those (39% and 43%, respectively) of serum starved fetal fibroblast cells reported by Zakhartchenko et al. (1999c) and Hill et al. (2000), although developmental potential of NT embryos was analogous to IVF-derived embryos (Table 3). Wells et al. (1999) showed that the development to term of cloned embryos was only one-quarter that of *in vitro* fertilized embryos. Studies on the quality of cloned embryos surviving to term should be carried out in the near future.

Mean nuclei numbers of NT blastocysts were smaller than those of IVF-derived blastocysts (Table 4), although there was no difference in developmental rate to blastocyst stage between groups (Table 3). These reduced nuclei numbers of NT embryos may be due to a reduction in the amount of oocyte cytoplasm occurring during enucleation (Westhusin et al., 1996; Peura et al., 1998). Therefore, low pregnancy rates and high abortion rates in NT embryos may be attributable simply to the reduced nuclei number (Yang et al., 1993). On the other hand, expanded NT blastocysts showed a higher nuclei number (103.8 ± 23.5, ranging from 62 to 137) than early NT blastocysts, which was similar to the result (ranging from 69 to 114 cells) of Kato et al. (1998).

It has been known that development of embryos produced by NT depends upon the maintenance of normal ploidy (Wilmut et al., 1997). To clarify whether NT blastocysts have normal chromosomes, we examined chromosome constitutions of blastocysts by a cytological analysis. All NT blastocysts showing metaphase spreads were male and had normal diploid. The results indirectly indicate that NT blastocysts produced in this study were derived from fetal fibroblast cells used as donor nuclei.

In conclusion, the results indicate that the NT oocytes fused by a single electric DC pulse of 1.8 kV/cm for 15 μsec have *in vitro* developmental potential to normal blastocysts like IVF-derived embryos.

V. REFERENCES

1. Baguisi, A., Behboodi, E., Melican, D. T., Pollock, J. S., Destrempes, M. M., Cammiso, C., Williams, J. L., Nims, S. D., Porter, C. A., Midura, P., Palacios, M. J., Ayres, S. L., Denniston, R. S., Hayes, M. L., Ziomek, C. A., Meade, H. M., Godke, R. A., Gavin, W. G., Overstrom, E. W. and Echelard, Y. 1999. Production of goats by somatic cell nuclear transfer. Nature Biotechnology, 17:456-461.
2. Bavister, B. B. and Ynangimachi, R. 1977. The effects of sperm extracts and energy sources on the motility and acrosome reaction of hamster spermatozoa *in vitro*. Biol. Reprod., 16:228-237.
3. Campbell, K. H. S., Loi, P., Cappai, P. and Wilmut, I. 1994. Improved development to blastocyst of ovine nuclear transfer embryos reconstructed during the presumptive S-phasr of enucleated activated oocytes. Biol. Reprod., 50: 1385-1393.
4. Campbell, K. H. S., McWhir, J., Richie, W. A. and Wilmut, I. 1996. Sheep cloned by nuclear transfer from a cultured cell line. Nature, 380: 64-66.
5. Cibelli, J. B., Stice, S. L., Golueke, P. J., Kane, J. J., Jerry, J., Blackwell, C., Ponce de Leon, A. and Robl, J. M. 1998. Cloned transgenic calves produced from nonquiescent fetal fibroblasts. Science, 280:1256-1258.

6. Hill, J. R., Winger, Q. A., Long, C. R., Looney, C. R., Thompson, J. A. and Westhusin, M. E. 2000. Development rates of male bovine nuclear transfer embryos derived from adult and fetal cells. Biol. Reprod., 62:1135-1140.
7. Kato, Y., Tani, T., Sotomaru, Y., Kurokawa, K., Kato, J., Doguchi, H., Yasue, H. and Tsunoda, Y. 1998. Eight calves cloned from somatic cells of a single adult. Science, 282: 2095-2098.
8. Kubota, C., Yamakuchi, H., Todoroki, J., Mizoshita, K., Tabara, N., Barber, M. and Yang, X. 2000. Six cloned calves produced from adult fibroblast cells after long-term culture. Proc. Natl. Acad. Sci., 97:990-995.
9. Miyoshi, K., Taguchi, Y., Sendai, Y., Hoshi, H. and Sato, E. 2000. Establishment of a porcine cell line from *in vitro*-produced blastocysts and transfer of the cells into enucleated oocytes. Biol. Reprod., 62:1640-1646.
10. Onishi, A., Iwamoto, M., Akita, T., Mikawa, S., Taketa, K., Awata, T., Hanada, H. and Perry, A.C.F. 2000. Pig cloning by microinjection of fetal fibroblast nuclei. Science, 289: 1188-1190.
11. Park, J. S., Han, Y. M., Lee, C. S., Kim, S. J., Kim, Y. H., Lee, K. J., Lee, K. S. and Lee, K. K. 2000. Improved development of DNA-injected bovine embryos co-cultured with mouse embryonic fibroblast cells. Animal Reproduction Science, 59:13-22.
12. Parrish, J. J., Susko-Parrish, J. L., Winer, M. A. and First, N. L. 1986. Capacitation of bovine sperm by heparin. Biol. Reprod., 38:1171-1180.
13. Peura, T. T., Lewis, I. M. and Trounson, A. O. 1998. The effect of recipient oocyte volume on nuclear transfer in cattle. Mol. Reprod. Dev., 50:185-191.
14. Prather, R. S., Stumpf, T. T., Rickords, L. F. 1992. Nuclear transplantation as a method of producing genetically identical livestock. Animal Biotechnology, 3:67-79.
15. Prather, R. S., Boice, M. L., Gibson, J., Hoffman, K. E. and Parry, T. W. 1995. *In vitro* development of embryos from sinclair miniature pigs: a preliminary report. Theriogenology 43:1001-1007.
16. Rosenkrans, C. F. Jr. and First, N. L. 1991. Culture of bovine zygotes to the blastocyst stage: Effects of amino acids and vitamins. Theriogenology, 35:266.
17. Schnieke, A. E., Kind, A. J., Ritchie, W. A., Mycock, K., Scott, A. R., Ritchie, M., Wilmut, I., Colman, A. and Campbell, K. H. S. 1997. Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. Science, 278:2130-2133.
18. Tsunoda, Y., Yasui, T., Nakamura, K., Uchida, T. and Sugie, T. 1986. Effect of cutting the zona pellucida on the pronuclear transplantation in the mouse. J. Exp. Zool., 240:119-125.
19. Vignon, X., LeBourhis, D., Chesne, P., Marchal, J., Heyman, Y. and Renard, J. P. 1999. Development of bovine nuclear transfer embryos reconstructed with quiescent and proliferative skin fibroblasts. Theriogenology, 51:216.
20. Wells, D. N., Misica, P. M., Day, A. M. and Tervit, H. R. 1997. Production of cloned lambs from an established embryonic cell line: A comparison between *in vitro*- and *in vitro*-matured cytoplasts. Biol. Reprod., 57:385-393.
21. Wells, D. N., Misica, P. M. and Tervit, H. R. 1999. Production of cloned calves following nuclear transfer with cultured adult mural granulosa cells. Biol. Reprod., 60:996-1005.
22. Westhusin, M., Collas, P., Marek, D., Sullivan, E., Stepp, P., Pryor, J. and Barnes, F. 1996. Reducing the amount of cytoplasm available for early embryonic development decreases the quality but not quantity of embryos produced

by *in vitro* fertilization and nuclear transplantation. Theriogenology, 46:243-252.

23. Wilmut, I., Schnieke, A. E., McWhir, J., Kind, A. J. and Campbell, K. H. S. 1997. Viable offspring derived from fetal and adult mammalian cells. Nature, 385:810-813.
24. Yang, X., Jiang, S., Farrell, P., Foote, R.H. and McGrath, A. B. 1993. Nuclear transfer in cattle: Effect of nuclear donor cells, cytoplast age, co-culture and embryo transfer. Mol. Reprod. Dev., 35:29-36.
25. Zakhartchenko, V., Alberio R., Stojkovic, M., Prelle, K., Schernthaner, W., Stojkovic, P., Wenigerkind, H., Wanke, R., Duchler, M., Steinborn, R., Muller, M., Brem, G. and Wolf, E. 1999a. Adult cloning in cattle: potential of nuclei from a permanent cell line and from primary cultures. Mol. Reprod. Dev., 54:264-272.
26. Zakhartchenko, V., Durcova-Hills, G., Schernthaner, W., Stojkovic, M., Reichenbach, H.D., Mueller, S., Steinborn, R., Mueller, M., Wenigerkind, H., Prelle, K., Wolf, E. and Brem, G. 1999b. Potential of fetal germ cells for nuclear transfer in cattle. Mol. Reprod. Dev., 52:421-426.
27. Zakhartchenko, V., Durcova-Hills, G., Stojkovic, M., Schernthaner, W., Prelle, K., Steinborn, R., Muller, M., Brem, G. and Wolf, E. 1999c. Effects of serum starvation and re-cloning on the efficiency of nuclear transfer using bovine fetal fibroblasts. J. Reprod. Fert., 115: 325-331.

요 약

태아 섬유아세포로 재구성된 핵치환 소 수정란의 체외발달

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본 연구는 태아 섬유아세포를 이용한 소 핵치환 난자의 융합에 있어서 여러 조건의 전기자극 처리 후 그에 따른 발달율을 조사하였다. 임신 45일령 태아로부터 섬유아세포를 분리하여 3~4 차례 계대배양한 후, 이들 단일 섬유아 세포들을 미세조작기를 이용하여 제핵된 난자의 위란강 내에 주입하였다. 본 실험에서는 첫째로, 다른 전기자극 조건의 적용에 따른 융합율 및 발달율을 비교 조사하였다. 180 V/mm의 조건하에서 다른 조건의 전기자극 시간 (15, 30, 45 μsec)을 처리하였을 때, 15 (45.5%, 120/264) 나 30 μsec (43.9 %, 106/241) 처리구가 45 μsec (23.2%, 58/250) 처리구 보다 높은 융합율을 나타내었다 ($P<0.05$). 그러나, 융합된 난자의 발달율에 있어서는 처리구 간에 차이가 나타나지 않았다. 다음 실험으로, 핵치환 난자의 체외발달에 있어서 15 μsec의 전기자극 시간의 조건하에서 다른 전장 (1.5, 1.8, 2.1 kV/cm)에 따른 효과를 조사하였다. 핵치환 난자의 융합이나 발달율에 전장에 따른 차이는 나타나지 않았다 ($P>0.05$). 마지막으로, 체세포 핵치환과 체외수정에 의한 수정란의 체외 발달율을 비교 조사하였다. 태아 섬유아세포로 재구성된 수정란의 배반포로의 발달율은 27.4% (75/274)로 나타났으며, 이는 체외수정에 의해 발달한 배반포의 비율 (24.5%, 58/237)과 유사하다는 것을 알 수 있었다. 그러나, 핵치환에 의해 유도된 배반포의 평균 세포 수는 체외수정에 의해 유도된 배반포 보다 낮게 나타났다. 결론적으로, 본 실험에서는 체세포로 핵치환된 소 난자의 전기적 융합에 대한 조건 (1.8 kV/cm, 15 μsec)을 확립하였다. 또한, 본 실험에서 나타난 결과들은 체세포로 재구성된 소 난자들이 비록 체외수정에 의해 생산된 배반포보다는 적은 세포수를 보여주었지만, 체외에서 정상적으로 배반포 단계까지 발달할 수 있다는 것을 제시하고 있다.

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