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Rabbit Cloning: Improved Fusion Rates Using Cytochalasin B in the Fusion Buffer

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ABSTRACT This study investigated the possibility of producing rabbits from embryos obtained by transfer of somatic cell nuclei; Muscle biopsies were taken from the upper part of the hind limb of fetuses at 24 days of gestation. Fetal fibroblasts were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% FCS at 38°C in 5% CO_2 in air. Cells were passaged at least three times; for 5 days prior to nuclear transfer, cells were cultured in 0.5% FCS medium to induce them into G_0. These cells then were placed into the perivitelline space of enucleated rabbit MII stage oocytes. Fusion media were Ca/Mg-containing 0.3 M mannitol (Buffer 1) or 0.3 M mannitol plus 7.5 μg/ml cytochalasin B (CB) (Buffer 2). Fusion was studied with 6 protocols: culture of donor cell-cytoplast complexes (CCCs) in TCM199 for 0, 30, or 60 min after micromaniupulation and electrical stimulation in Buffer 1 or Buffer 2. Fusion rates were 57.4, 42.9 and 52.8%, respectively, in groups 0 + B1, 30 + B1, and 60 + B1, significantly lower than the rates in groups 0 + B2, 30 + B2 and 60 + B2 (75.9, 75.7, and 76.4%, respectively) ($P < 0.05$). Thus, CB in electrical fusion buffer improved the fusion rate. The percentages of blastocyst formation were 40% in 0 + B2 and 37.1% in 0 + B1 groups, significantly higher ($P < 0.05$) than those in 30 + B1 (20%), 30 + B2 (15.6%), 60 + B1 (13%) and 60 + B2 (8.3%). A total of 653 cloned embryos were at 1-cell, 2–4-cell, or morula/blastocyst stages were transferred to 44 mated or non-mated synchronized recipients. No cloned embryos developed to term. *Mol. Reprod. Dev. 61: 187–191, 2002.* © 2002 Wiley-Liss, Inc.

Key Words: cloning; rabbit; embryo; somatic cells; fusion

INTRODUCTION

To generate the first offspring from nuclear transfer in mammals, nuclei of undifferentiated embryonic cells isolated as blastomeres before the blastocyst stage, or from inner cell mass cells of blastocysts were used (Willadsen, 1986; Smith and Wilmut, 1989; Collas and Barnes, 1994; Keefer et al., 1994). Demonstration of nuclear totipotency of cultured embryonic differentiated cells was obtained using cultured cell lines (Campbell et al., 1996; Schnieke et al., 1997; Wells et al., 1997). Finally, there has been much progress in producing offspring from somatic cell nuclear transfer in sheep (Wilmut et al., 1997), mice (Wakayama et al., 1998; Zhou et al., 2000), cattle (Kato et al., 1998; Wells et al., 1999; Vignon et al., 1998), goats (Baguisi et al., 1999), and pigs (Onishi et al., 2000; Polejaeva et al., 2000). Although embryonic nuclear transfer was reported in rabbits a decade ago (Stice and Robl, 1988; Collas and Robl, 1990; Yang et al., 1992), no obvious progress in the somatic cell nuclear transfer has been reported, although there have been several attempts (Dinnyes et al., 1999; Galat et al., 1999; Lagutina et al., 2000).

Successful fusion of somatic cells with oocytes is more difficult than with embryonic cells (Dominko et al., 1999), due to the reduced area of contact between donor cell and cytoplast (Prather et al., 1987; Sims and First, 1994; Wells et al., 1997); average fusion rates between 50% (Keefer et al., 1994; Kato et al., 1998) and 65% have been reported (Wells et al., 1997; Kato et al., 1998). In this study, we developed a new buffer to improve fusion rates, and transferred 653 nuclear transplanted embryos at different stages into synchronized recipient rabbits.

MATERIALS AND METHODS

Cell Cultures

Biopsies were obtained from Chinchilla rabbit fetuses at 24 days of gestation. Muscle biopsies (about 2 mm^3) were taken from the upper part of the hind limb of the fetuses. Tissues were cut into pieces and digested with 0.25% trypsin (Gibco, Life Technologies, USA) for

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30 min at 37°C. The digested cells and tissues were cultured in DMEM/F12 (1:1) (Gibco, Life Technologies) supplemented with 20% fetal bovine serum (FBS, Gibco, Life Technologies, USA) in a 5% CO_2 in air incubator. Cells were passed when 70~80% of them converged. Cells passed 3–5 generations were used as donors. The serum concentration was decreased to 0.5% to starve the donor cells 3–5 days before the nuclear transfer.

Animal Superovulation and Oocyte Collection

The oocyte donors were Japanese big eared white rabbits (6–12-month-old) obtained from the Animal Breeding Center, Institute of Zoology (Academia Sinica, China). The rabbits were caged and housed in pairs. Superovulation and oocyte collection were as previously described (Chen et al., 1999). Briefly, rabbits were superovulated by FSH and hCG treatment. A total of 0.6–0.8 mg FSH (Institute of Zoology, Chinese Academy of Sciences) was injected intramuscularly twice daily for 3 days, and 100 IU hCG was injected 72 hr after the first FSH injection. Oocytes were collected from oviducts 15–17 hr after hCG injection. The cumulus oocytes complexes (COC) were placed in TCM-199 supplemented with 0.5% hyaluronidase (Sigma, St. Louis, MO) for 3–5 min, and gentle pipetting was performed to remove the cumulus cells. A total of 97 rabbits were used as oocyte donors and 2358 oocytes were obtained in this study.

Nuclear Transfer Procedure

Enucleation. Matured oocytes were incubated in M2 medium (Sigma, St. Louis, MO) with 7.5 μg/ml cytochalasin B (CB) for 15 min, and then enucleated by aspirating the first polar body and the metaphase II spindle plate in a small amount of surrounding cytoplasm with a 20–25 μm outer diameter glass pipette. The oocytes had been previously stained in TCM199 medium containing 10% FCS, 5 μg/ml Hoechst 33342 (Sigma, St. Louis, MO), and 7.5 μg/ml cytochalasin B (Sigma, St. Louis, MO) for 5 min. Confirmation of successful enucleation was achieved by visualizing the karyoplast under ultroviolet light (Chen et al., 1999).

Preparation of donor cells. Quiescent donor cells were obtained by culture in medium with 0.5% FCS for 3–5 days to induce them into G_0 phase (Wilmut et al., 1997; Chen et al., 1999). Immediately prior to injection, a single cell suspension was prepared by digestation with 0.25% trypsin, and resuspended and maintained in DMEM/F12 supplemented with 0.5% FCS until injection. Cells used for nuclear transfer in these experiments were between passages 3 and 5 of culture.

Microinjection. The microinjection process was as described by Chen et al. (1999) and Li et al. (2000). The pipette was introduced through the same slit in the zona pellucida made during enucleation, and the cell was wedged between the zona and the cytoplast membrane to facilitate close membrane contact for subsequent fusion.

Fusion and Activation

To study fusion rates, the following treatments were used: after injection, the cell-cytoplast complexes (CCCs) were washed three times in TCM199 containing 10% FCS and held in the same medium at 38°C, 5% CO_2 in air for 0, 30, or 60 min prior to fusion in Buffer 1 or Buffer 2. Buffer 1 was comprised of 0.3 M mannitol, 0.5 mM calcium and 0.1 mM magnesium; Buffer 2 was Buffer 1 plus 7.5 μg/ml CB. Fusion was performed at room temperature in a chamber with two stainless steel electrodes 1 mm apart overlaid with fusion buffer. The CCCs were manually aligned with a fine pipette so that the contact surface between the cytoplast and the donor cell was parallel to the electrodes. Cell fusion was induced with two DC pulses of 1.5 kV/cm for 80 μs each, delivered by a Kefa Cell Electrical fusion system (Academia Sinica). Fusion rates were determined 30–60 min after the fusion pulses. At this time, the fused oocytes were again electrically stimulated (0.8 kV/cm DC pulse for 40 μs) and then cultured in TCM 199 containing 10 μg/ml cycloheximide (Sigma, St. Louis, MO) for 4 hr to ensure activation.

In Vitro Culture of Nuclear Transplanted Embryos

The nuclear transferred embryos were cultured in 100 μl drops of TCM199 supplemented with 10% FCS at 38°C, 5% CO_2 in air. Embryos were checked for cleavage every 24 hr.

Evaluation of Cell Numbers in Blastocysts

Numbers of cells in nuclear transferred blastocysts were assessed 96 hr after culture by preparation of embryos for chromosome analysis (Tarkowski, 1966).

Embryo Transfer

Mature Japanese big eared white rabbits were used as recipients. Recipients were either treated (1) the same as oocyte donors but with a half dosage of FSH and hCG and stimulation of the recipient cervix with a sterile glass rod (Non-Mated); or (2) synchronized by natural mating with male Japanese big ear white rabbits (Mated). Either 1-cell, 2–4-cell, or morula-blastocyst stages nuclear transplanted embryos were transferred to the oviducts of the recipient rabbits.

Experimental Designs

Experiment 1. This experiment was replicated five times to examine effect of CB in fusion buffer on fusion rate and development of the nuclear transplanted embryos. CCCs were fused in 6 groups: 0 + B1, 0 + B2, 30 + B1, 30 + B2, 60 + B1, and 60 + B2 as described above. Most of the fused oocytes were cultured to observe their development, the others were transferred into the recipients. Since the best blastocyst rates were obtained in groups 0 + B1 and 0 + B2, which were used in subsequent experiment for fusion.

Experiment 2. This experiment was replicated twenty six times to produce nuclear transplanted

embryos for embryo transfer. Fusion protocol was 0 + B1 and 0 + B2. The nuclear transplanted embryos at the 1-cell, 2–4-cell, or morula/blastocyst stages were transferred into the recipients.

Statistical Analysis

Differences between experimental groups were analyzed using Student's *t*-test or with Chi-square using the Fisher-Yates Correction.

RESULTS

Effects of Cytochalasin B on Fusion and Reconstructed Embryo Development

After micromanipulation, the CCCs were cultured 0, 30, or 60 min in TCM 199 and then placed directly into Buffer 1 or Buffer 2 for electrical stimulation (Table 1). Fusion rates were 57.4, 42.9, and 52.8%, respectively, in groups 0 + B1, 30 + B1, and 60 + B1, significantly lower ($P < 0.05$) than the rates in groups 0 + B2, 30 + B2 and 60 + B2 (75.9, 75.7, and 76.4%, respectively). CB in the fusion buffer clearly improved the fusion rate. The percentages of blastocyst formation were 40% in 0 + B1, and 37.1% in 0 + B2 groups, significantly higher ($P < 0.05$ or 0.01) than those in the other groups (Table 1). Cell numbers of the blastocysts ranged from 80 to 95, with no significant differences among treatments. Therefore, we used 0 + B1 and 0 + B2 fusion treatments for a second experiment in which embryos were transferred to recipients.

Results of Embryo Transfer

A total of 653 cloned embryos were transferred to 44 recipients (Table 2). No cloned rabbit was born with any treatment.

DISCUSSION

Cloning Rabbits From Embryonic Donor Cells

Bromhall (1975) transferred nuclei from morula-stage embryos to intact, mature recipient oocyte in which the metaphase chromosomes were not removed; only a few of these reconstructed embryos developed in vitro to the morula stage. These embryos were most likely tetraploid and their limited development was not unexpected. Stice and Robl (1988) produced the first six genetically verified nuclear transplant rabbits with donor nuclei from 8- or 16-cell embryos and enucleated mature recipient oocytes. Collas and Robl (1990) modified the procedures of nuclear transplantation: 10% of the nuclear transplanted embryos (8–16-cell-stage donor nuclei) (21% of those transferred to recipients) developed to offspring; these rates were similar to the development of nonmanipulated control embryos. Blastomeres from 8-cell embryos cultured for 20–24 hr to the 32–64-cell stage were used as nuclear donor cells by Yang et al. (1992); a total of 243, 2–4-cell

TABLE 1. Effects of CB in Fusion Buffer on Fusion and Reconstructed Embryo Development (Summaries of 5 Replicates)

Time*	Buffer1	Buffer2	CCCs	Fusion (%)	Embryos cultured**	Cleavage (%)	Morula (%)	Blastocyst (%)	Cells per blastocyst
0	+		108	62 (57.4)^a	35	24 (68.6)^b	18 (51.4)^b	14 (40)^a	82^a (65∼96)
0		+	112	85 (75.9)^b	62	52 (83.9)^a	39 (62.9)^a	23 (37.1)^a	93^a (79∼111)
30	+		105	45 (42.9)^a	30	15 (50)^b	10 (33.3)^c	6 (20)^b	93^a (67∼101)
30		+	103	78 (75.7)^b	32	26 (81.3)^a	15 (46.9)^b	5 (15.6)^b	80^a (79∼111)
60	+		53	28 (52.8)^a	23	—	5 (21.7)^c	3 (13.0)^c	86^a (35∼127)
60		+	72	55 (76.4)^b	36	24 (66.7)^b	13 (36.1)^c	3 (8.3)^c	95^a (46∼142)

Values with different superscripts in the same row differ, a,b; b,c: $P<0.05$, a,c: $P<0.01$.
*Time between the end of microinjection and transfer to buffer for electric pulse.
**The other fused nuclear trasplanted embryos were transferred into recipients.

TABLE 2. Development of Cloned Embryos Following Transfer Into Recipients

Fusion treatment	Type of recipient	Number of recipients	Stage transferred	Number of embryos transferred	Number born
0 + Buffer 1	Non-mated	11	1-cell	205	0
	Non-mated	5	Morula/blastocyst	51	0
	Mated	8	1-cell	146	31*
	Mated	4	Morula/blastocyst	49	10*
0 + Buffer 2	Non-mated	6	2–4-cell	86	0
	Non-mated	3	Morula/blastocyst	26	0
	Mated	4	2–4-cell	61	11*
	Mated	3	Morula/blastocyst	29	15*
Total		44		653	66

*All of the baby rabbits were Japanese big eared white rabbits.

embryos were transferred to 15 pseudopregnant recipients resulting in 8 young (3%).

Fusion Efficiency When Cloning Mammals

The results in Table 1 indicate that addition of CB to the electrical buffer improved rates of fusion. Cytochalsin B is often used in current nuclear transferprocedures, Yang et al. (1992) examined the effect of CB on activation of rabbit oocytes; oocytes were placed into a preincubated micromanipulation droplet containing 7.5 μg/ml CB for approximately 1 hr and then were exposed to fusion medium (0.3 M mannitol) followed by electric pulse (0.1–0.15 kV/cm, 1.0 MHz AC current for 5–15 sec to align the oocytes, then a DC pulse of 2.4 kV/cm for 60 μs). Exposure of oocytes to CB for 1 hr, followed by activation treatment, significantly improved oocyte development into blastocysts compared with oocytes activated without pre-exposure (38 vs. 26%, $P < 0.05$). Smith and Wilmut (1989) found that incubation of nuclear transferred sheep embryos in CB for 1 hr after electrical stimulation improved development to morula and blastocyst stages. Collas and Robl (1990) confirmed this result in rabbits. The improved development was probably due to reduced fragmentation between 16-cell and 64-cell stages, when CB was used. Smith and Wilmut (1989) found an improvement in electrofusion when AC current was included, especially for donor cells with a small inner cell mass. Electrofusion rates were higher with ovine16-cell blastomeres (82%) than with ICM cells (47%). The addition of CB (7.5 μg/ml) to the medium for 1 hr after elertrofusion significantly improved development to the morula-blastocyst stage of reconstituted embryos derived from both 16-cell blastomeres (11 vs. 35%) and ICM cells (0 vs. 56%), indicating that the cytoskeletal mechanisms operating at the time of oocyte activation may affect the developmental potential of exogenous nuclei. CB effectively disrupts the microfilament component of the cytoskeleton and prevents cytokineses (Siracusa et al., 1980).

The improved development of reconstructed embryos placed in CB medium for 1 hr immediately after the fusing pulse suggests that when eggs were not placed with inhibitors, the donor nucleus may have been affected by microfilament-dependent cytoskeletal mechanisms operating at the time of oocyte activation, possibly causing haploidization or other aneuploidy defects.

Cloning Rabbit From Somatic Donor Cells

The fusion protocols used in this study were beneficial to development of reconstructed embryos (Table 1). Blastocyst development rates were 40 and 37.1% in groups 0 + B1 and 0 + B2, respectively, which were higher than those of other reports (Dinnyes et al., 1999; Galat et al., 1999; Lagutina et al., 2000). Unfortunately, none of the cloned embryos developed to term after embryo transfer, even though development in vitro to the morula/blastocyst stage was high, and the cell numbers of blastocysts were from 80.4 to 94.75, which compared favourably to control blastocysts derived from embryonic donor cells (91 ± 10.2), or in vivo developed embryos (Table 2) (106 ± 5.1) (Stice and Robl, 1988).

The reason for failure to develop to term is not clear, but some may think that it is related to secretions of the rabbit oviduct and uterus. Embryos developed in vivo are walled with a thick mucin coat, which may be important for implantation. In this study, fertility of the mated recipients was acceptable, so this was not the problem.

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