Effect of Cytochalasin B in Activation Medium on the Development of Rat Somatic Cell Nuclear Transfer Embryos

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ABSTRACT

This study was conducted to evaluate the effect of cytochalasin B (CB) treatment in the activation medium on the development of somatic cell nuclear transfer (SCNT) rat embryos. Fetal fibroblast cells were isolated from a Day 14.5 fetus, and the oocytes for recipient cytoplasm were recovered from 4-week old Sprague Dawley rats. After enucleation and nuclear injection, the reconstructed oocytes were immediately exposed to activation medium consisting of 10 mM $SrCl_2$ with or without CB for 4 hr, and formation of pseudo-pronucleus (PPN) was checked at 18 hr after activation. Then, they were transferred into day 1 pseudopregnant recipients (Hooded Wistar) or cultured for 5 days to check their developmental competence *in vivo* or *in vitro*. The number of PPN was not affected by CB treatment during the activation. However, CB treatment supported pre-implantation development of rat SCNT embryos. Embryos generated by the procedures of SCNT were also capable of implanting, with 1 implantation scar found from a recipient following the transfer of 87 SCNT embryos to four foster mothers. The result of the present study shows that rat SCNT embryo can develop to post-implantation stage following treatment with CB.

(Key words : Somatic cell nuclear transfer, Oocyte activation, Implantation, Cytochalasin B treatment, Rat)

INTRODUCTION

Transgenic rat would be useful as an experimental animal model for medical research along with transgenic mice. All transgenic rats have been produced by DNA microinjection into the pronuclei of zygote with fewer than 20 transgenic rat lines generated (Charreau *et al.*, 1996). Transgenic animals have also been produced by nuclear transfer (NT) using genetically modified somatic donor cells (Schnieke *et al.*, 1997; Cibelli *et al.*, 1998; Baguisi *et al.*, 1999; Park *et al.*, 2001; Hyun *et al.*, 2003). Although there is one report on the production of rats following somatic cell NT (SCNT; Zhou *et al.*, 2003), other attempts to produce cloned rat embryos from NT using embryonic and somatic cells have largely had limited success (Kono *et al.*, 1988; Hayes *et al.*, 2001; Iannaccone *et al.*, 2001; Kato *et al.*, 2001; Du *et al.*, 2002; Jiang *et al.*, 2002; Roh, 2005).

In rats, a high rate of spontaneous activation occurs in ovulated oocytes during *in vitro* culture. Auto activated oocytes extrude the second polar body within 60 to 90 min of culture and show scattered chromosomes, a state termed metaphase III (MIII). After reaching this MIII state, oocytes exhibit very low rates of normal cleavage following induced activation (Keefer and Schuetz, 1982). This represents a major obstacle for SCNT in the rat, as control of activation is a crucial step for successful SCNT. To overcome this incomplete and abortive activation, Zhou *et al.* (2003) used a protease inhibitor that reversibly stabilizes most oocyte MII metaphases for up to 3 hr and obtained two cloned pups after SCNT. In the previous studies, the author suggested that oocytes must be activated within 2 hr after collection from donor animals (Roh *et al.*, 2003), and the reconstructed embryos should be activated immediately after nuclear injection in rat SCNT programme (Roh, 2005). It was also proposed that artificial activation is intentionally delayed after SCNT to induce premature chromosome condensation (PCC; Wilmut *et al.*, 1997; Wakayama *et al.*, 1998; Wells *et al.*, 1999), which is believed to be an essential step for reprogramming of donor nucleus, especially in ungulate SCNT programme. Immediate activation after nuclear injection procedure was also successful in mice (Wakayama and Yanagimachi, 2001). Immediate activation after injection method does not need cytoskeletal inhibitor treatment as the reconstructed oocyte bypasses PCC and forms one large pseudo-pronucleus (PPN) without pseudopolar body (PPB) extrusion. However, the effect of the

* This work was supported by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MOST) (No. M10641000001-06N4100-00110).

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presence of cytoskeletal inhibitor during the activation is not clear in rats because their oocytes may be activated spontaneously during or after nuclear injection.

Although Zhou *et al.* (2003) cloned rat by SCNT, the protocol they used has not been reproduced by other scientists yet. Therefore rat cloning procedure may be still in suboptimal condition. In the present study, it was evaluated that the effect of the presence of cytochalasin B during the activation immediately after nuclear injection on the developments of rat SCNT embryos.

MATERIALS AND METHODS

Reagents and Media

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise stated. All the media used here were based on modified rat embryo culture medium (mR1ECM; Miyoshi *et al.*, 1997). Embryo manipulations were carried out in Hepes-buffered mR1ECM (HR1ECM) at room temperature in the air.

Recovery of Oocytes

Four-week old Sprague Dawley (SD) rats were superovulated by intraperitoneal injections of 10 IU equine chorionic gonadotropin (eCG) and 10 IU human chorionic gonadotropin (hCG) (Intervet, NSW, Australia) given 48 hr apart. Superovulated females were killed by cervical dislocation 14 hr after hCG injection, and the oviducts were removed and transferred into a Petri dish containing 2 ml HR1ECM supplemented with 300 IU/ml hyaluronidase (bovine testis). The oviduct ampullae were opened, and the cumulus-enclosed oocytes were released. After 2~3 min exposure to the medium, the cumulus-free oocytes were washed twice in HR1-ECM before enucleation.

Donor Cell Preparation

Fetal fibroblast cells were obtained from a Day 14.5 SD rat fetus and cultured in DMEM (GIBCO BRL, Grand Island, NY, USA) supplemented with 10% FBS (Gibco BRL). For each passage, cells were cultured until confluent, were disaggregated by incubation in a 0.25% trypsin solution for 1 min at 37℃, and were allocated to three new dishes containing 10% FBS supplemented DMEM for further passaging. For long- term storage, the cells were collected after trypsinizatiopn, frozen in 10% dimethyl sulfoxide, and were stored in liquid nitrogen.

Enucleation and Nuclear Injection

Enucleation was carried out by slitting the zona pellucida in the region of the cytoplasmic bulge, using a micro-needle with subsequent aspirating of the bulge containing metaphase plate through the slit with the holding pipette (Fig. 1). The enucleated oocytes were then placed in mR1ECM prior to nuclear injection. Nuclei were injected into enucleated oocytes, using pipettes with an approximate inner diameter of 5 μm, which were back-loaded with mercury and coated with 6% polyvinylpyrolidone immediately before use. The pipette with isolated nucleus was inserted through the slit in the zona pellucida and advanced three-quarters of the way through the oocyte. Piezoelectric actuation was used to break the membrane, and the nucleus was deposited.

Activation, *In Vitro* Culture and Embryo Transfer

Reconstructed oocytes were immediately exposed to activation medium consisting of 10 mM $SrCl_2$ with or without 5 μg/ml cytochalasin B in mR1ECM for 4 hr (ActCB+ vs ActCB-, respectively). Following this activation, the oocytes were cultured in mR1ECM at 37℃ under mineral oil in the atmosphere of 5% CO_2 in air. Some SCNT embryos were checked for PPN formation at 18 hr after activation and were cultured *in vitro* for 5 days. Their cleavage was monitored at 44 hr and *in vitro* developmental rates were observed at 119 hr after activation, respectively. After *in vitro* culture, the embryos were stained by Hoechst 33342 (5 mg/ml) for 10 min and checked under ultraviolet light to examine the number of nuclei of the embryos. Some SCNT embryos were transferred into Day 1 pseudopregnant recipients (Hooded Wistar) with 10~20 embryos in each oviduct. The pregnancy of recipient rats were checked by wei-

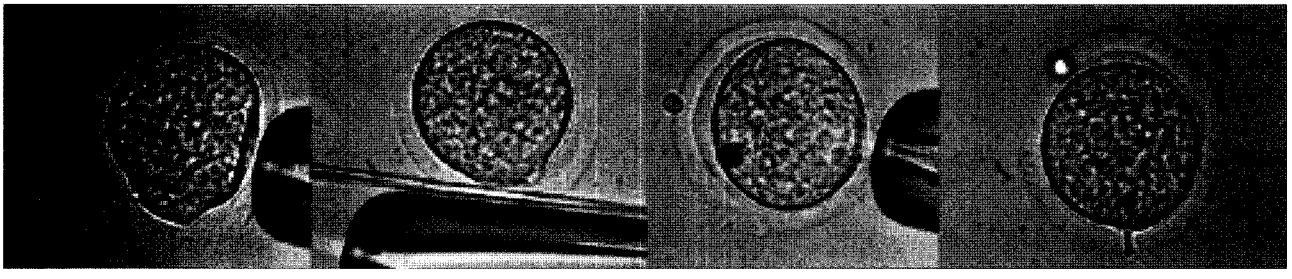

Fig. 1. Enucleation of rat oocytes. After slitting, the bulge containing metaphase plate was aspirated with the holding pipette.

ghting the rats everyday after 13 days of embryo transfer, and the uteri of recipient rats were recovered between Day 18~20 of embryo transfer. Then, the evidence of pregnancy was examined.

Statistical Analysis

Experiment was repeated four times. Differences in the mean percentages of pronuclear formation, cleavage and further embryonic development among the treatments were analyzed by Fischer's exact probability test. A value of $p<0.05$ was accepted as an indication of statistical significance.

RESULTS

The embryos in groups ActCB+ and ActCB-formed PPN at the rate of 69.3% (70/144) and 70.8% (51/103) respectively (Table 1). No PPB extrusion was found in both groups. The rate of cleavage to the 2-cell stage was significantly higher in ActCB+ group (45.1%, 32/71) than ActCB- one (33.9%, 19/56). One early blastocyst with small blastocoelic cavity with 11 nuclei was found only in ActCB+ group (Table 2). The reconstructed embryo from ActCB+ group was also capable of implanting, with 1 implantation scar found from a recipient following the transfer of 87 SCNT embryos to four foster mothers (Table 3).

Table 3. Embryo transfer results of rat somatic cell nuclear transfer embryos following activation with cytochalasin B

Embryo transfer into oviduct (left/right)	Implantation scar (s)*	Pups born
16/0	-	-
14/13	-	-
18/0	1	-
14/12	-	-

* Eighteen to twenty days after embryo transfer.

DISCUSSION

In the present study, SCNT embryos, derived using rat fetal fibroblast cell nuclei, were developed *in vitro* and *in vivo*. Only one *in vitro* embryos developed to the blastocyst stage and the evidences of one implantation scar from *in vivo* embryos were found in this experiment. Apart from the one successful report on cloned rat production by SCNT (Zhou *et al.*, 2003), development of rat embryos has been largely unsuccessful (Hayes *et al.*, 2001; Iannaccone *et al.*, 2001; Kato *et al.*, 2001; Jiang *et al.*, 2002). Before the first cloned rat production by Zhou *et al.* (2003), the evidence of implantation after transferring rat SCNT oocytes into foster mother was reported by the other group. (Du *et al.*, 2002). However, *in vitro* culture system for rat SCNT embryos are still inefficient. There are only two reports on the production of morulae and blastocysts derived from *in vitro* cultured rat SCNT embryos (Iannaccone *et al.*, 2001, Roh, 2005). To overcome this technical problem, the embryos are usually transferred directly to rats or mice, as foster mothers or intermediate hosts respectively, without further culture *in vitro* after SCNT (Hayes *et al.*, 2001; Kato *et al.*, 2001; Hira-

Table 1. Effect of cytochalasin B (CB) in activation medium on pseudo-pronuclear formation after nuclear injection and activation

Goups*	No. of reconstituted oocytes	Survived after activation (%)	PPN** formed (%)	1 PPN	2 PPNs
ActCB+	144	101(70.1)	70(69.3)	61	9
ActCB-	103	72(69.9)	51(70.8)	49	2

* ActCB+(−), activation supplemented with (without) cytochalasin B.

** PPN, pseudo-pronucleus.

Table 2. Effect of cytochalasin B (CB) in activation medium on *in vitro* development of rat somatic cell nuclear transfer embryos

Goups*	Survived after activation	Cleavage (%)	4-Cells (%)	Blastocysts (%)
ActCB+	71	32(45.1)[a]	9(12.7)	1(1.4)
ActCB-	56	19(33.9)[b]	6(10.7)	-

* ActCB+(-), activation supplemented with (without) cytochalasin B.

[a,b] $p<0.01$.

bayashi *et al.*, 2003). Iannaccone *et al.* (2001) successfully produced rat NT blastocysts (8~12%) from *in vitro* culture using cytochalasin B supplemented medium for nuclear injection. However, It was argued previously that due to cytoplasmic elasticity after cytoskeletal inhibitor treatment, it was not possible to inject a cell nucleus into rat oocyte cytoplasm in cytochalasin B supplemented medium as it usually resulted in cell lysis (Roh, 2005). Certain unknown factor(s) or know-how(s) between the two protocols might affect *in vitro* development of rat SCNT embryos.

In previous studies, the author suggested that rat MII oocytes must be activated within 2 hr of cervical dislocation to avoid abortive spontaneous activation of rat MII oocytes (Roh *et al.*, 2003). The author also stated that rat SCNT oocytes did not show any evidence of NEBD or PCC before activation and no multiple PPN was found after activation regardless of their activation timing (Roh, 2005).

Wakayama and Yanagimachi (2001) demonstrated that immediate activation after nuclear injection procedure was also successful in mice, however, they did not treat cytoskeletal inhibitor during the activation process because PCC phenomenon did not happen and PPB was not extruded as well when reconstructed oocytes were activated immediately after nuclear injection. However, the present study showed that treatment of cytoskeletal inhibitor during the activation supported *in vitro* development of rat SCNT embryos, although they were activated immediately after the injection of donor nuclei. Temporal disruption of cytoskeleton by cytochalasin B might interrupt abnormal cytokinesis induced by spontaneous activation and possibly support parthenogenetic development of reconstructed rat oocytes.

Since our enucleation process showed complete elimination of metaphase II chromosomes (100%, data not shown), implantation scar after embryo transfer was believed to be from SCNT oocytes, not from the parthenogenetic one. As enucleated oocytes without any nuclear materials can not develop beyond post-implantation stage, the evidence of implantation scar revealed post-implantation development of rat SCNT embryo, although any fetal material was not found in uteri. However, the protocol demonstrated here could not support full-term development of rat SCNT embryos, and further studies are needed to develop SCNT programme in this important species.

In conclusion, cytochalasin B treatment partially supports pre-implantation development of rat SCNT embryos, and the embryos generated by the procedures of SCNT can develop to the post-implantation stage.

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(Received: 18 May 2007/ Accepted: 15 June 2007)