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Effect of the Timing of Oocyte Activation on Development of Rat Somatic Cell Nuclear Transfer Embryos

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ABSTRACT

Methods for activation of reconstructed oocytes were examined for the production of nuclear transfer (NT) rat embryos using fetal neural stem cells as donor. Neural stem cells were isolated from Day 14.5 rat fetuses, 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 $SrCl_2$ for 4 h (immediate activation after injection; IAI), or cultured *in vitro* for 2~3 h before activation treatment (injection before activation; IBA). Pre-activated oocytes were also used for NT to test reprogramming potential of artificially activated oocytes. The oocytes were grouped as IIA (immediate injection after activation) and ABI (activation 2~3 h before injection). Following NT, the oocytes were cultured *in vitro*. Development of the NT embryos was monitored at 44 and 119 h after activation. The embryos in groups IAI, IBA, and IIA were cleaved to the 2-cell stage at the rates of 36.6% (15/41), 39.5% (17/43) and 46.3% (25/54), respectively. However, in the ABI group, only one embryo (1.8%, 1/55) was cleaved after activation. After *in vitro* culture, two NT embryos from IAI group had developed to the morula stage (4.9%; 2/41). However, no morula or blastocyst was obtained in the other groups. These results suggest that immediate activation after injection (IAI) method may be used for the production of rat somatic cell NT embryos.

(Key words : Nuclear transfer, Oocyte activation, Rat)

INTRODUCTION

Transgenic rat would be a 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 produced (Charreau *et al.*, 1998). Embryonic stem cell technology, which is routinely used to generate targeted mutation in the mouse, has not yet been established in the rat limiting their genetic manipulation. Transgenic animals have also been produced by nuclear transfer (NT) using genetically modified somatic donor cells [Baguisi *et al.*, 1999, Cibelli *et al.*, 1993, Hyun *et al.*, 2003, Park *et al.*, 2001, Schnieke *et al.*, 1997). 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 [Hayes *et al.*, 2001; Iannaccone *et al.*, 2001; Jiang *et al.*, 2002; Kato *et al.*, 2001; Kono *et al.*, 1988).

In rats, a high rate of spontaneous activation occurs in ovulated oocytes during *in vitro* culture (Keefer and Schuetz, 1982). 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. This represents a major obstacle for SCNT in the rat, as control of activation is a crucial step for successful NT. 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 h and obtained two cloned pups after SCNT. In the previous study, the author suggested that oocytes must be activated within 2 h after collection from donor animals in order to provide suitable recipient cytoplasm for a NT programme since attempts to induce artificial activation of oocytes cultured *in vitro* for more than 2 h was unsuccessful (Roh *et al.*, 2002).

In mice and ungulate NT, artificial activation is inten-

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tionally delayed after NT to induce premature chromosome condensation (PCC; Wakayama *et al*., 1998; Wells *et al*., 1999; Wilmut *et al*., 1997) which is believed to be an essential step for reprogramming of donor nucleus. In mice (Wakayama *et al*., 2001) and pigs (Hyun *et al*., 2003), however, normal cloned animals were also produced by immediate activation without PCC induction following NT using G0/G1 stage somatic cell nuclei as donors. So, in some species, inducing PCC may not be a crucial step for SCNT. Since spontaneous activation phenomenon is more critical for further development of rat oocytes, delayed activation may not be a suitable method in rat SCNT programme.

In the present study, to investigate the optimal timing of oocyte activation in the rat NT programme, four oocyte activation protocols were evaluated with different time point of activation by assessing nuclear status and *in vitro* development of rat NT 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 mR1-ECM (HR1ECM) at room temperature in 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 h apart. Superovulated females were killed by cervical dislocation at 14 h after hCG injection and the oviducts 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 HR1ECM before enucleation.

Nuclear Donor (Neural Stem Cell) Preparation

Neural stem cells were isolated from Day 14.5 SD rat fetuses in neurobasal media (GIBCO BRL, Grand Island, NY, USA) in the presence of bFGF (10 ng/mL) and EGF (10 ng/mL; Johe *et al*., 1996). These neural stem cell clones differentiated to multiple fates: neurons, astrocytes, and oligodendrocytes.

Enucleation and Nuclear Injection

Enucleation was carried out by slitting the zona pellucida in the region of the cytoplasmic bulge using a microneedle with subsequent sucking of the bulge containing metaphase plate through the slit with the holding pipette. 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 mm, which were back-loaded with mercury and coated with 5% polyvinylpyrrolidone immediately before use. The pipette with isolated nucleus was inserted through the slit in the zona pellucida and advanced between half and three-quarters of the way through the oocyte. Piezoelectric actuation was used to break the membrane and the nucleus was deposited. Enucleation and nuclear injection were performed in cytochalasin B-free medium.

Activation and *In Vitro* Culture

Reconstructed oocytes were immediately exposed to activation medium consisting of 10 mM $SrCl_2$ with 5 μg/mL cytochalasin B for 4 h (immediate activation after injection; IAI), or cultured in mR1ECM for 2～3 h before activation treatment (injection before activation; IBA). Pre-activated oocytes were also used to test whether artificial activation prior to NT could avoid abortive activation of rat oocytes. These oocytes were treated as follows; (1) A part of oocytes were exposed to the activation medium consisting of 10 mM $SrCl_2$ without cytochalasin B and enucleation was performed in the same medium. After total 1 h of exposure to $SrCl_2$, neural stem cell nuclei were injected into enucleated oocytes. These oocytes were grouped as IIA (immediate injection after activation). (2) The other oocytes were activated for 4 h in the activation medium with cytochalasin B and enucleation was also performed in the cytochalasin B added HR1ECM to prevent cell disruption, and these NT oocytes were grouped as ABI (activation before injection). Detailed experimental design is described in the Fig. 1. Following this activation the oocytes were cultured in mR1ECM at 37 ℃ under mineral oil in an atmosphere of 5% CO_2 in air. Half of the NT embryos were checked for pronuclear formation the following morning and the rest of embryos were monitored their cleavage and *in vitro* developmental rates at 44 and 119 h after activation, respectively. After *in vitro* culture, the NT embryos in all experiments 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. When checking pronuclear formation a small and opaque swollen nucleus, quite different from intact somatic cell nucleus, in the NT oocytes was termed a small pseudo-pronucleus (S-PPN). A large and typical swollen nucleus of an activated NT oocyte was considered as large pseudo-pronucleus (L-PPN; Fig. 2). The embryos with L-PPN had more than one nucleolus whereas the embryos with S-PPN showed only one

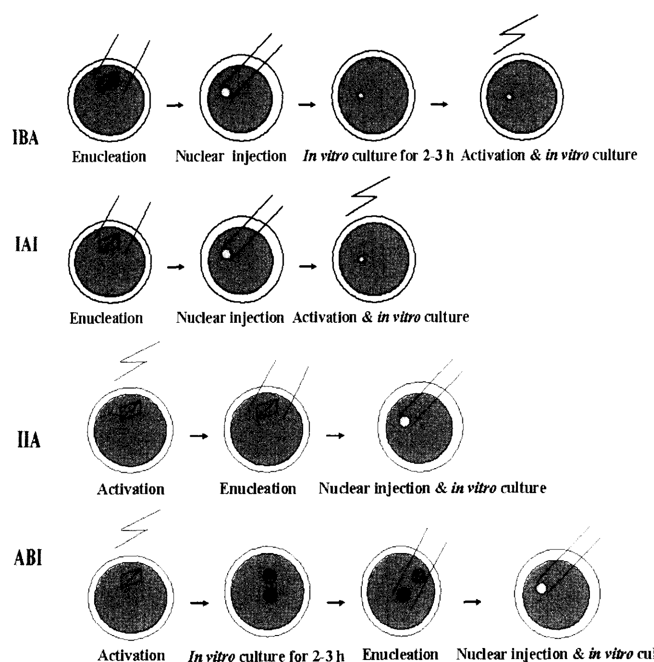

Fig. 1. Four activation protocols: from the top, immediate activation after injection (IAI), injection before activation (IBA), immediate injection after activation (IIA) and activation before injection (ABI).

nucleolus in their nuclei.

Animal Ethics

All animal experiments were approved and performed under the Monash University Animal Experimentation Ethics Committee guidelines, which follow those specified in the National Research Council (NRC) publication 'Guide for Care and Use of Laboratory Animals' (National Research Council. 1996).

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 IAI, IBA, and IIA cleaved to the 2-cell stage at the rate of 36.6% (15/41), 39.5% (17/43) and 46.3% (25/54), respectively. However, in ABI group, only one embryo (1.8%, 1/55) was cleaved after activation (Table 1). Higher L-PPN formation (51.2%, 21/41; Fig. 3) was obtained in NT embryos of IAI group ($p<0.05$). Morula stage embryos were also only found in IAI group embryos (4.9%, 2/41) and their respective cell numbers were 8 and 9 (Fig. 2 & Table 1).

Fig. 2. ***In vitro*** **development of rat NT embryos.** (a) a NT embryo with a large pseudo pronucleus (L-PPN) after activation, (b) 2-cell stage NT embryos, (c) a partially compacted morula with 9 nuclei.

Table 1. ***In vitro*** **development of rat nuclear transfer (NT) embryos following parthenogenetic activation with four different protocols**

Goup*	No. of NT embryos in culture	2-cells≤ (%)	4-cells≤ (%)	Morulae (%)
IAI	41	15 (36.6)[a]	10 (24.4)	2 (4.9)
IBA	43	17 (39.5)[a]	5 (11.6)	-
IIA	54	25 (46.3)[a]	6 (11.1)	-
ABI	55	1 (1.8)[b]	-	-

*IAI: immediate activation after injection, IBA: injection before activation.
IIA: immediate injection after activation, ABI: activation before injection.
[ab]$p<0.01$.

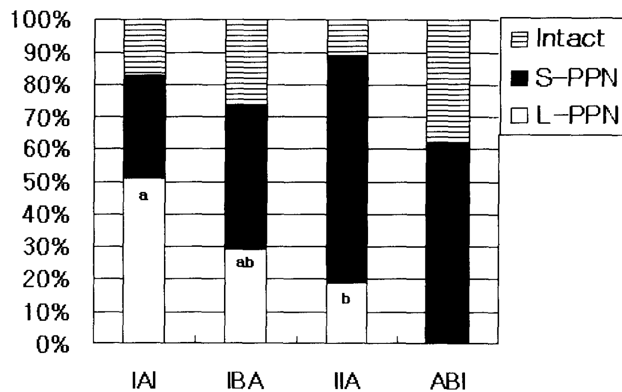

Fig. 3. Pseudo-pronuclear formation patterns of somatic cell nuclei after nuclear transfer. (a) L-PPN: large pseudo-pronucleus, S-PPN: small pseudo-pronucleus, Intact nucleus: nucleus with no size change (no nuclear swelling), (b) Higher L-PPN formation (51.2%, 21/41) was obtained in nuclear transfer embryos of IAI group than others (IIA: 19.1%, 9/47; $^{ab}p<0.05$). There was no significant difference between IBA (29.3%, 12/41) and other two groups (IBA vs IAI: $p\approx 0.1$, IBA vs IIA: $p\approx 0.5$). No L-PPN was obtained from ABI group. No multiple PPN-formed NT oocytes were found in all groups.

DISCUSSION

In the current study, NT embryos, derived using rat neural stem cell nuclei were cultured *in vitro* and some embryos developed to the morula/blastocyst stage. These embryos only had 8~9 cell nuclei and showed premature compaction, suggesting better culture conditions for rat SCNT embryos need to be developed. Apart from the one successful report of cloned rat production by SCNT (Zhou *et al.*, 2003), development of rat embryos have been largely unsuccessful (Hayes *et al.*, 2001; Iannaccone *et al.*, 2001; Jiang *et al.*, 2002; Kato *et al.*, 2001). Since *in vitro* culture system for rat NT embryos are still inefficient, NT embryos are usually transferred directly to rats or mice, as foster mothers or intermediate hosts respectively, without further culture *in vitro* after NT (Hayes *et al.*, 2001; Hirabayashi *et al.*, 2003; Kato *et al.*, 2001). Hayse *et al.* (2001) obtained some rat NT blstocysts after culturing embryos in the uterus of a rat. To the best of author's knowledge, there is only one report on production of blastocysts derived from *in vitro* cultured rat NT embryos (Iannaccone *et al.*, 2001). However, Jiang *et al.* (2002) reported that no rat NT embryo was developed beyond the 2-cell stage *in vitro*. In the present study, we were also not able to produce rat NT blastocysts *in vitro*. Iannaccone *et al.* (2001) successfully produced rat NT blastocyst from *in vitro* culture using cytochalasin B, a cytoskeletal inhibitor, supplemented medium for nuclear injection. However, other groups (Hayes *et al.*, 2001; Hirabayashi *et al.*, 2003; Jiang *et al.*, 2002; Kato *et al.*, 2001) used cytochalasin B-free medium for nuclear injection. Also in preliminary experiments of the present study, 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. So, certain unknown factor(s) between the two protocols may affect blastocyst development of rat NT embryos.

In a previous study, the author suggested that rat MII oocytes must be activated within 2 h of cervical dislocation to avoid abortive spontaneous activation of rat MII oocytes (Roh *et al.*, 2002). Since MII chromosomes of oocytes were removed within 2 h of cervical dislocation in our NT programme, spontaneous activation of enucleated oocytes might be different from intact ones. Hence the author compared delayed activation and immediate activation after nuclear injection (IBA and IAI, respectively). In both IBA and IAI groups, oocytes were enucleated within 2 h after cervical dislocation to reduce potential MII chromosomal effect(s) on spontaneous activation of rat oocytes (Keefer and Schuetz, 1982). In addition to this, if spontaneous activation is a critical factor in rat NT, use of pre-activated oocytes may be an alternative way for rat SCNT. As pre-activated oocytes can be fused to blastomeres in any cell cycle stage, pre-activation of enucleated oocytes before NT had been widely used for embryonic cell (blastomere) NT in domestic animals (Campbell *et al.*, 1993; Stice *et al.*, 1994). However, because only cytoplast from MII stage oocyte developed to term after SCNT, pre-activated oocyte are not used for somatic cell cloning in most mammals.

To test reprogramming potential of pre-activated rat oocytes for SCNT, oocytes were activated first then, enucleation and nuclear injection were performed. In one group, enucleation and nuclear injection were performed immediately after start of activation stimulus (IIA). In the other group, activated oocytes by exposure to $SrCl_2$ with cytochalasin B for 4 h were enucleated (ABI). At this stage, normally two pronuclei were observed in the oocytes. Then, somatic cell nuclei were injected.

As shown in Fig. 3 and Table 1, higher L-PPN formation and embryonic development were shown in NT embryos of IAI group, although *in vitro* culture results were not statistically different. Different from SCNT in other species including sheep and mice (Wilmut *et al.*, 1997; Wakayama *et al.*, 1998), IAI method tended to be more efficient for rat NT. In addition, different from mouse NT embryos (Wakayama *et al.*, 1998; Wakayama *et al.*, 2001), rat NT embryos in IBA group did not show PCC after nuclear injection and multiple PPNs after activation. Nuclear envelope breakdown (NEBD) followed by PCC is generally shown in NT oocytes when introduced cell nuclei were exposed to MII oocytes which contain high level of MPF activity (Campbell *et al.*, 1993). However, in this study, NT oocytes did not show any evidence of NEBD or PCC before activation (data not shown). So, the author concluded that spontaneous activation may affect the competence of *in vitro* development of rat oocytes even after enucleation

and injection before oocyte activation (IBA) was not suitable to rat NT, which is commonly used for SCNT in other mammals (Wakayama *et al.*, 1998; Wells *et al.*, 1999; Wilmut *et al.*, 1997).

Interestingly, IIA showed similar developmental competence to IBA and approximate 90% of nuclei injected to activated oocytes in IIA group were swollen in the oocytes (Fig. 3), and cleavage rate also tended to be higher than other groups (Table 1). However, most of swollen nuclei in IIA group were S-PPN which may be regarded as evidence of incomplete reprogramming, and the developmental rate over 4-cell stage of these embryos was comparably lower than that of embryos in IAI group. This result demonstrated that pre-activation of recipient oocyte was not applicable to rat somatic cell NT programme regardless of nuclear injection timing, although nuclear swelling and cleavage rates of IIA group embryos were comparable to those of IAI and IBA group ones.

In conclusion, immediate activation after injection (IAI) method might be one of the ways to overcome abortive activation in rat somatic cell NT, and may be applied to produce transferable rat NT embryos. However, to increase the outcomes of rat NT, additional studies are required to further improve the efficiency of the procedure.

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