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Comparison of Developmental Efficiency of Murine Somatic Cell Nuclear Transfer Protocol

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ABSTRACT

The Somatic cell nuclear transfer (SCNT) method can be applied to various fields such as species conservation, regenerative medicine, farming industries and drug production. However, the efficiency using SCNT is very low for many reasons. One of the troubles of SCNT is that it is highly dependent on the researcher's competence. For that reason, four somatic cell nuclear injection methods were compared to evaluate the effect of hole-sealing process and existence of cytochalasin B (CB) on efficiency of murine SCNT protocol. As a results, the microinjection with the hole-sealing process, the oocyte plasma membrane is inhaled with injection pipette, in HCZB with CB was presented to be the most efficient for the reconstructed in SCNT process. In addition, we demonstrated that the oocytes manipulated in Hepes-CZB medium (HCZB) with CB does not affect the developmental rate and the morphology of the blastocyst during the pre-implantation stage. For this reason, we suggest the microinjection involving hole-sealing in HCZB with CB could improve SCNT process efficiency.

(Key words: Somatic cell nuclear transfer (SCNT), Developmental efficiency, Cytochalasin B (CB))

INTRODUCTION

The somatic cell nuclear transfer (SCNT) method was developed because of the necessity of applications in species conservation, regenerative medicine, farming industries and drug production. Eventually, patient-specific embryonic stem cells had been studied so that they could be created manually, and it was established within mice (Munsie *et al.*, 2000) and primates (Byrne *et al.*, 2007). More recently, embryonic stem cells were established using human adult SCNT (Chung *et al.*, 2014; Yamada *et al.*, 2014). After the success of Wilmut and Campbell cloning sheep (Wilmut *et al.*, 1996), the late 1990s saw the use of SCNT in cloning mice (Wakayama *et al.*, 1998) and bovine (Kato *et al.*, 1998). However, despite the murine embryo culture and manipulation part being easy, the efficiency for successful SCNT embryos was low due to many technical and biological problems. Traditionally, domestic animal SCNT embryos are successfully developed by electrofusion for insertion donor cell nucleus into oocyte. In addition, murine oocyte is so fragile therefore, it can be easily lysed by electrofusion or conventional microinjection (Wakayama *et al.*, 2006). To improve SCNT efficiency, the piezo-assisted microinjection method is introduced by Wakayama and colleagues (Wakayama *et al.*, 1998). This technique was first set up in the University of Hawaii, and it is called the "Honolulu technique". However, this method still has limitations in performing murine SCNT procedures. It depends on the hands skills of researchers, thus it is difficult to produce the same results from each of the researchers from different labs to produce the same results. For this reason, based on this technique, some groups established different somatic cell injection techniques protocols suitable to their own laboratory conditions. One of the techniques is the hole-sealing, the oocyte plasma membrane is inhaled with injection pipette, after somatic cell injection in medium with Cytochalasin B (CB) immediately (Jaenish *et al.*, 2008). In addition, there were many effects to further improve the efficiency of SCNT by using chemicals. One significant study is that actin polymerization inhibitors like Trichostatin A (TSA) and Latruculin A (Lat A), which are histone deacetylase inhibitors, showed positive effects and have been used widely (Wakayama *et al.*, 2006; Wakayama *et al.*,

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2012). In recent years, the injection of Kdm4d mRNA improves SCNT efficiency over 90% by downregulating H3 lysine 9 trimethylation (H3K9me3) (Zhang *et al.*, 2014). However, technical problems still remain such as cytosol stress, membrane damage and researcher skill proficiency. Despite diverse problems of the technique, the mouse SCNT has multifarious advantages for studying developmental biology including reprogramming, imprinting, early embryonic development and regenerative medicine (French *et al*., 2008; Noggle *et al*., 2011). Here, we compare development efficiencies of several kinds of the somatic cell injection method.

MATERIALS and METHODS

1. Chemicals

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

2. Animals and culture of embryos.

6-8 weeks old C57BL6 X DBA2 F1-hybrid (B6D2F1) female mice offered for metaphase Ⅱ (MⅡ) oocytes. All of culture and handling media rested on CZB and KSOM media. The control oocytes and reconstructed oocytes were activated in calcium free-CZB media with 10 mM SrCl2 and 5 μg/ml CB for 5 h 30 min. Then, the activated oocytes were cultured in KSOM culture media for 4 days. All animal experiments were approved under the agreement guidelines of the Institutional Animal Care and Use Committee of Seoul National University (Approval number: SNU-130123-5-5)

3. Collection oocytes and preparation of donor cells.

The 7.5 IU equine chorionic gonadotropin (eCG, Daesung Microbiology Lab. CO., Ltd, Korea) injected female B6D2F1 mice by intraperitoneal for superovulation and 7.5 IU human chorionic gonadotropin (hCG, Daesung Microbiology Lab. CO., Ltd, Korea) after 48 h later. Oocytes were collected 15 h after hCG injection, and the oviducts were removed and transferred to 2 ml TCM-199 with Earl's salts medium (TCM-washing) supplemented with 300 IU/ml hyaluronidase. After tearing the ampullae of the oviducts, the oocytes were released with cumulus cells. After 3 to 5 min exposure to the hyaluronidase-containing TCM-washing medium, the cumulus-free oocytes were washed five times in Hepes-CZB medium (HCZB) before micro manipulation. The cumulus cells were suspended in a 10 μl TCM-washing droplet was mixed with 12% polyvinylpyrrolidone (PVP), and the dish was covered with mineral oil.

4. Enucleation

12 to 15 oocytes were manipulated in a 10 μl droplet of HCZB containing 10 μg/ml CB, which had been placed under mineral oil and the 37℃ operation heat chamber on a microscope stage. The zona pellucida of the oocyte was pierced by several piezo pulse using a piezo-actuated micromanipulator (PMM-150FU, Prime Tech Ltd., Ibaraki, Japan). The diameter of enucleation pipette is 6 to 8 μm. The MⅡ chromosome-spindle complex was removed with a small amount of ooplasm using an enucleation pipette. After enucleation, the enucleated oocytes were moved into HCZB for washing three times, kept for up to 30 min in KSOM culture medium.

5. Nuclear injection

In a PVP-TCM washing droplet, cumulus cells were collected by 5 μm diameter injection pipettes and aspirated in and out several times for membrane breaking. After donor cell collection, zona pellucida was drilled out by piezo pulse. Then, the donor cells in the pipette were introduced into the enucleated oocytes by the same piezo pulse. To demonstrate the effects of hole-sealing process and existence of CB on the developmental efficiency of SCNT embryos, four experimental groups were designed as described in Table 1. After SCNT, all of reconstructed oocytes were washed three times in 37℃ HCZB after injection and kept for 10 min room temperature HCZB for stabilizing oocytes. After stabilization, reconstructed

Table 1. The four different donor cell injection methods.

Group	Hole-sealing	CB treatment in HCZB
Method 1	X	X
Method 2	O	O
Method 3	X	O
Method 4	O	X

oocytes were kept in KSOM culture medium for up to 30 min in humidified 37℃ incubator.

Also see the SCNT procedure video. Link to: https://youtu.be/j-1vgEc3A8c

6. Activation and in vitro culture

All reconstructed mouse oocytes were kept to a calcium free-CZB with 5 μg/ml CB and 10 mM SrCl2 (ACZB) for oocyte activation. After 5 h 30min, activated oocytes were washed in HCZB three times and transferred in KSOM culture medium for in vitro culture during 120 h.

7. Statistical analysis

All experiments were iterated three times. All percentage data obtained in this study are presented as mean ± standard deviation (S.D.). Rates of preimplantation embryo development and production of reconstructed oocytes were analyzed by ANOVA using Prism software 5.0 (GraphPad Software, Inc.). A probability of $P<0.05$ was considered significant.

RESULTS

The comparison between two types of donor cell injection methods is shown in Fig. 1. The cutting zona pellucida and producing a crack in the plasma membrane of oocyte were the same for both methods. However, the injection pipette was removed rapidly after donor cell injection in Method 1 and Method 3 (Fig. 1A), whereas the small amount of cytosol of oocytes were inhaled by the injection pipette using hole-sealing process after donor cell injection in Method 2 and Method 4 (Fig. 1B).

The yield rates of reconstructed oocytes from SCNT oocytes were shown different by each other injection methods in Table 2. Method 2 gave the highest yield rate for reconstructed oocytes (96%) followed by Method 1 (68.3%), Method 3 (15%) and Method 4 (10%). The yield rate of reconstructed oocytes using

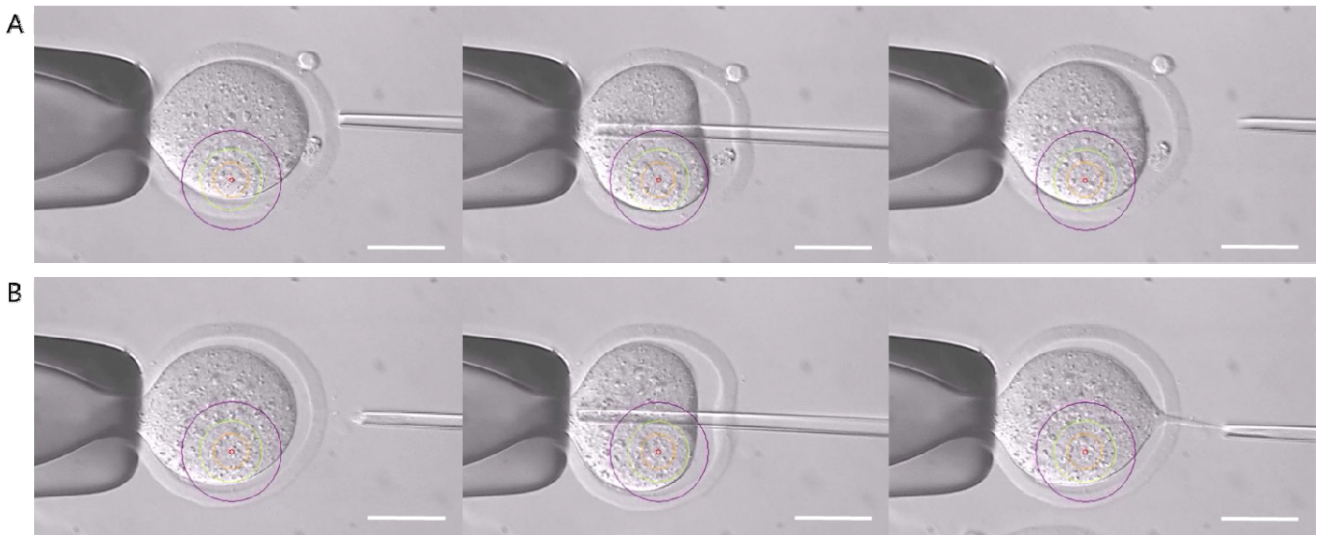

Fig. 1. Two types of donor cell injection methods. (A) Donor cell injection without hole-sealing process. (B) Injection with hole-sealing process using pipette. Scale bar = 20 μm.

Table 2. The yield of successful reconstruction of SCNT oocytes using four different injection methods

Group	Total donor cell injected oocytes	Reconstructed oocytes (%)
Method 1	139	95 (68.3 ± 4.4)
Method 2	101	97 (96.0 ± 15.8)*
Method 3	100	15 (15.0 ± 2.4)**
Method 4	100	10 (10.0 ± 3.7)**

This experiment was replicated three times. Data are the mean ± S.D.

*, **Different superscripts denote a significant difference compared with other groups ($P<0.05$).

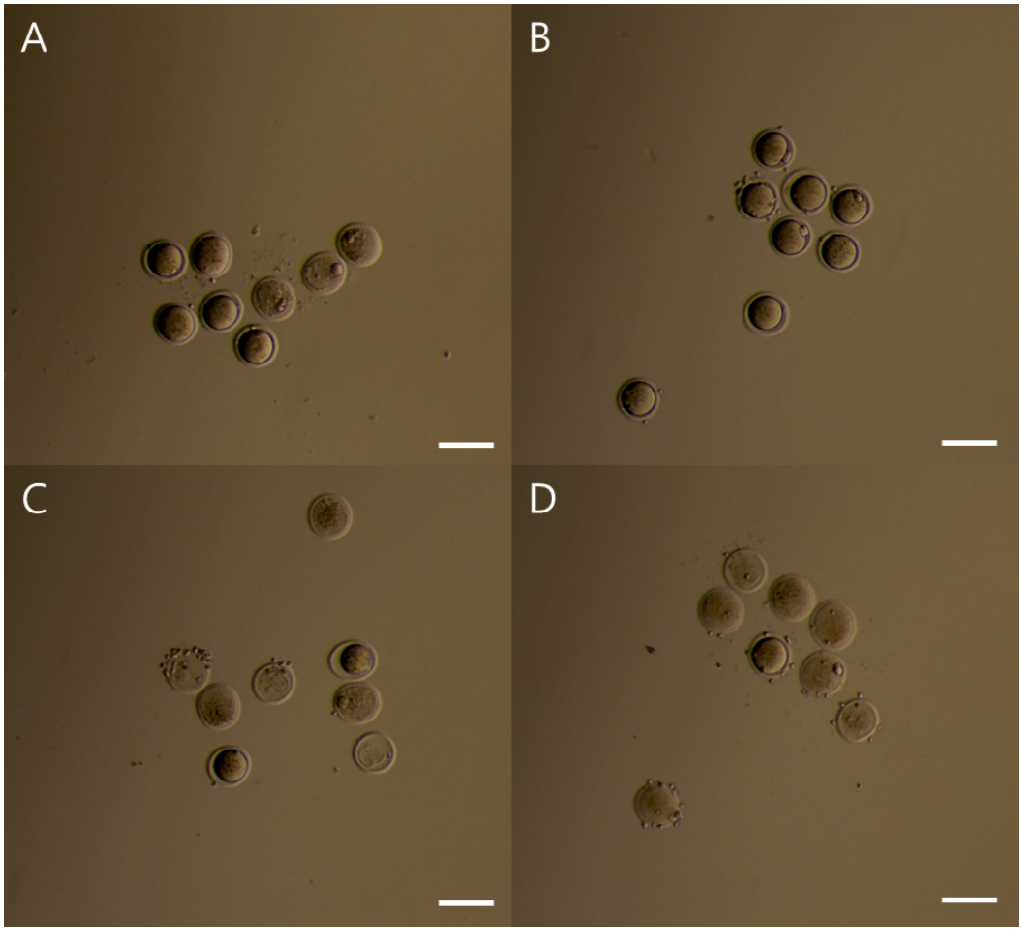

Fig. 2. The reconstructed oocytes after donor cell injection by (A) Method 1, (B) Method 2, (C) Method 3 and (D) Method 4. Scale bar = 50 μm.

Table 3. Developmental rates of reconstructed oocytes using two different injection methods.

	No. of embryos developed to (%)			
Group	Reconstructed oocytes	2 cells	4 cells	Blastocysts
Method 1	95	40 (42.1 ± 3.0)	22 (23.2 ± 1.5)	18 (19.0 ± 1.3)
Method 2	97	46 (47.4 ± 3.9)	29 (30.0 ± 2.9)	22 (22.7 ± 2.3)

Method 3 and Method 4 was significantly low compared to Method 1 and Method 2.

After nuclear injection, SCNT oocytes were transferred to HCZB in room temperature for oocyte membrane stabilization. In this process, damaged oocytes were lysed. The oocytes proceeded by Method 3 (Fig. 2C) and Method 4 (Fig. 2D) showed significantly high numbers of lysed oocytes compared with Method 1 (Fig. 2A) and Method 2 (Fig. 2B). For this reason, Method 1 and Method 2 were compared during the next step.

The developmental rate of SCNT embryo of Method 1 and Method 2 is shown in Table 3. There were no significant differences between Method 1 and Method 2 in 2-cell (42.1 ± 3.0 vs. 47.4 ± 3.9), 4-cell (23.2 ± 1.5 vs. 30.0 ± 2.9) and blastocyst (19.0 ± 1.3 vs. 22.7 ± 2.3) stages.

The morphology of the blastocysts was shown in Fig. 3 after SCNT using Method 1 (Fig. 3A) and Method 2 (Fig. 3B). There were also no significant differences in the cell numbers and the diameters of the blastocysts as well as their hatching rates.

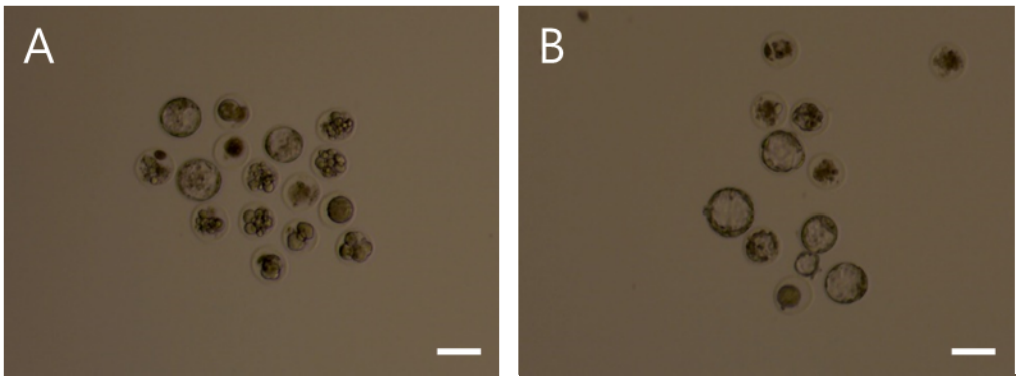

Fig. 3. The morphology of SCNT blastocysts derived from (A) Method 1, and (B) Method 2. Scale bar = 50 μm.

DISCUSSION

In previous studies, it was known that CB should not be used for the donor cell injection in SCNT (Wakayama *et al.*, 1998). However, some groups applied existing SCNT protocol using the CB-added medium during donor cell insertion (Egli and Eggan, 2006; Jaenisch *et al.*, 2008). In this study, four different injection methods were compared. In the nuclear injection in HCZB with CB (Method 3), it failed to reconstruct most of the time. However, in the same condition with Method 2, the hole-sealing added-protocol using the injection pipette showed the highest efficiency above all things in the HCZB including CB medium condition (Method 2).

The failure of Method 3 might be caused by CB, which disintegrates F-actin organization under the membrane and induces lysis of oocytes with small crack for donor cell injection (MacLean-Fletcher and Pollard, 1980). In this case, the membrane without F-actin cannot recover the crack. Therefore, the pipette assisted hole-sealing is effective for producing reconstructed oocytes.

The method with injection hole-sealing in HCZB without CB (Method 4) mostly failed to produce reconstructed oocytes. It suggests that a lot of damage is exerted by pipette inhalation on the oocyte membrane, especially when CB does not exist under the conditions. Consequently, it leads to breaking the oocyte membrane immediately. For the above reasons, the efficiency of producing reconstructed oocytes was very low in Method 3 and Method 4. Therefore, only the developmental rates of reconstructed oocytes by Method 1 and Method 2 from 1-cell to blastocyst stages were compared. As a result, there were no significant differences in the development rate of Method 1 and Method 2. In addition, blastocyst formation and morphology showed no significant difference.

The collecting of oocytes and producing SCNT embryos is demanding because lots of money, time and exertion of researchers is often required. For these reasons, it is important to increase the efficiency of producing SCNT embryos. In this study, the yield of reconstructed oocytes was significantly different between Method 1 (68.3%) and Method 2 (96.0%), while CB does not affect developmental rates of reconstructed oocytes in pre-implantation stage. Hence, the yield of SCNT murine embryos by hole-sealing process is comparably higher than conventional one.

In conclusion, these results suggest that somatic cell injection with the hole-sealing process in HCZB including CB could produce reconstructed oocytes with high efficiency. Furthermore, we demonstrated that CB in the manipulating medium does not affect embryo development significantly.

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