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Nuclear Transfer by Using a Laser-Assisted Zona Pellucida Piercing Technique in Mice

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Somatic cells nuclear transfer (SCNT) is a useful tool in studies of developmental biology and animal cloning. However, SCNT experiments only are allowed to skilled technical experts. In this experiment, laser-assisted zona pellucida piercing tool (LASER) was applied in murine SCNT. LASER minimized the use of piezo-driven micromanipulator (PIEZO), reducing chances of problems caused by PIEZO pulses. LASER reduced time that took to pierce zona pellucida in removal of nucleus from oocyte and somatic cell injection, which might have taken longer time with PIEZO. Time and difficulties that took researcher of equivalent skilled for their experiments were decreased with LASER, and this might affect the improvement of embryonic development. (LASER, 6.2% versus PIEZO, 2.9%; $P< 0.05$). Thus, these data support that the use of LASER can be used for zona pellucida piercing in murine SCNT program as an alternative to PIEZO.

Key words: mouse, nuclear transfer, zona pellucida piercing, laser

Introduction

Cloning animals have been generated using adult somatic cells in many species for the last 10 years. In addition, autologous male and female embryonic stem cells from somatic cell nuclear transfer (SCNT) embryos were produced (Wakayama *et al.*, 2005). This technology can be applied to autologous stem cell therapy in regenerative medicine area. However, animal cloning is still inefficient. Especially, success rate of cloned mice from SCNT is very low. The percentage of live progeny obtained from murine SCNT in reports is < 2% (Wakayama *et al.*, 2005). It is known that many factors such as donor cell cycle, oocytes activation, manipulation time, physical damage and chemical agents affect development of pre-implantation embryos *in vitro* (Kishigami *et al.*, 2007).

Many researchers improve developmental rate by addition of chemical agents such as DMSO (Wakayama *et al.*, 2001) or Trichostatin A (Kishigami *et al.*, 2007). However, another critical factor, physical damage by manipulation was not focused to improve SCNT protocol. In general, a specialized machine called piezo-driven micromanipulator (PIEZO) is needed to inject somatic cell nucleus into cytoplast in mouse SCNT (Wakayama *et al.*, 1998, 1999) although some investigators who experienced difficulties setting up PIEZO also produced cloned mice without PIEZO (Zhou *et al.*, 2000; Rybouchkin *et al.*, 2002). The PIEZO is used three times in SCNT, zona-drilling during enucleation and donor cell insertion, and cytoplasmic membrane puncture for donor cell injection. Without this device, weak membrane of mouse oocyte is likely to burst away during the injection of somatic cells. However, only well-trained technicians can control PIEZO for zona pellucida drilling or nuclear injection (Kishigami *et al.*, 2007). In the present study, we minimized the use of PIEZO device to reduce physical damage during micromanipulation process by using new laser-assisted piercing device, called XYClone™ (Hamilton Thorne Biosciences, Beverly, MA, U.S.A.; LASER). LASER is generally used in infertility clinic for zona pellucida puncture (Tadir and Doughlas-Hamilton, 2007) and mammalian subfertility

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researches (Anzai *et al.*, 2006; Allegra *et al.*, 2007). Chen *et al.* (2004) also reported that the laser is easy and stable method for making a zonal opening, although they used different type of diode laser (Fertilase™, Medical Technologies, Montreux SA, Switzerland). However, they did not use PIEZO after laser-assisted zonal puncture.

In this study, we used LASER for zona pellucida puncture to insert enucleation and injection pipettes into perivitelline space without PIEZO pulses then used PIEZO for somatic donor nuclear injection. Success rate of enucleation, nuclear injection and development to the blastocyst stage were examined to evaluate the use of LASER in the murine SCNT program.

Materials and Methods

Animal and Chemicals

Six-week-old C57BL6 X DBA2 F1-hybrid (B6D2F1) female mice were used to recover oocytes and cumulus cells. All inorganic and organic compounds were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.) unless otherwise stated.

Media

All embryos were cultured in a KSOM medium (Lawitts and Biggers, 1991) at 37.5°C under 5% CO_2 in air. Oocyte manipulations were carried out in Hepes-buffered CZB (HCZB; Chatot *et al.*, 1989).

Collection of oocytes and cumulus cells

Female B6D2F1 mice were superovulated by intraperitoneal injections of 7.5 IU eCG and 7.5 IU hCG, given 48 h apart. Oocytes were recovered 13 to 15 h after hCG injection, and the oviducts were removed and transferred into a Petri dish containing 2 ml HCZB, supplemented with 300 IU/ml hyaluronidase. The oviduct ampullae were opened and the cumulus-enclosed oocytes were released. After 2 to 3 min exposure to the medium, the cumulus-free oocytes were washed twice in HCZB before micromanipulation. Cumulus cell suspension in hyaluronidase-containing HCZB was transferred to HCZB, containing 10% (w/v) polyviny- lpyrrolidone (PVP), and kept at room temperature for up to 2 h before injection (Wakayama *et al.*, 2001).

Enucleation

Ten to fifteen oocytes were transferred to a 4 μl droplet of HCZB containing 5 μg/ml cytochalasin B, which had been placed previously under mineral oil in the operation chamber on the microscope stage. In the first experimental group, zona pellucida of the oocyte was 'drilled' by applying several PIEZO pulses to the tip of an enucleation pipette with 7-10 μm diameter (Wakayama *et al.*, 1998). The metaphase II chromosome–spindle complex (MII-CX) was drawn into the pipette with a small amount of accompanying ooplasm and removed from the oocytes. After oocytes enucleated, they were transferred into KSOM and kept for up to 30 min before nuclear injection (grouped as PIEZO). In the second experimental group, zona pellucida of the oocyte was 'punched' by LASER. Then, MII-CX with a small amount of accompanying ooplasm was removed (grouped as LASER).

Nuclear injection

Nuclear injection was carried out about 30 min to 1.5 h after enucleation. In a droplet of HCZB containing 10% PVP, a cumulus cell was drawn in injection pipette with 5-7 μm diameter and donor cell move in and out until the plasma membrane was broken. Then, the pipette moved to cytochalasin-free HCZB droplet for nuclear injection. The pipette containing the donor cells was directly injected into the enucleated oocytes through cytoplast membrane by 1 or 2 PIEZO pulse(s). Zona pellucida puncture before nuclear injection was performed by either PIEZO or LASER. After the injection, the reconstructed embryos were remained in medium before activation. All micromanipulation processes were performed at room temperature.

Activation and *in vitro* culture

Nuclear transferred oocytes were exposed to an activation medium, consisting of 10 mM $SrCl_2$ with 5 μg/ml cytochalasin B in calcium-free CZB for 5 h, and then cultured for 120 h in KSOM at 37°C in a humidified 5% CO_2 in air. The medium was pre-incubated for 2 h in a CO_2 incubator before culturing activated oocytes. Success rate of enucleation, nuclear injection and development to the blastocyst stage were examined.

Statistical analysis

Each experiment was replicated at least four times. Enucleation and injection success, cleavage and further embryonic development between treatments were compared by Chi-square test using SPSS software package (Version 12.0; SPSS Inc., Chicago, IL, USA). Differences were considered statistically significant for P values < 0.05.

Animal ethics

All animal experiments were approved and performed under the guidelines of the Institutional Animal Care and Use Committee of Seoul National University.

Results and Discussion

Successful nuclear injection, cleavage and blastocyst formation rates in LASER were higher than those in PIEZO ($p < 0.05$; Table 1).

Table 1. *In vitro* development of nuclear transfer murine embryos enucleated and nuclear injected using a laser-assisted piercing technique

Piercing method[a]	Number of oocytes	Nuclear injection success (%)	Cleavage (%)	Blastocysts (%)
LASER	210	120 (57.1)[b]	24 (11.4)[b]	13 (6.2)[b]
PIEZO	204	91 (44.6)[c]	14 (6.9)[c]	6 (2.9)[c]

[a]LASER: laser-assisted zona pellucida piecing; PIEZO: zona pellucida drilling by piezo-driven micromanipulator.
[b,c]Within a column, values without a common superscript differed ($P < 0.05$).

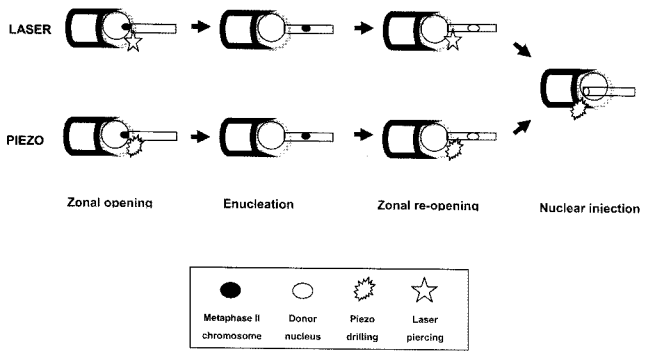

Fig. 1. Illustrations of enucleation and injection using laser beams (LASER) or piezo-actuated micromanipulator (PIEZO). LASER: A hole on zona pellucida was made by laser beam then the enucleation pipette was passed through the hole for entering into the oocytes. Metaphase II chromosome-spindle complex was then aspirated out. For nuclear injection, the injection pipette containing a donor nucleus passed through the hole made by laser beam then the pipette was advanced onto ooplasm close to the 9-O'clock position. One piezo pulse was applied to break the oolemma then the nucleus was released. PIEZO: The enucleation pipette penetrated zona pellucida by one or several piezo pulses then aspirated chromosome-spindle complex of the oocytes. For injection, the injection pipette containing a nucleus penetrated zona pellucida by piezo pulses. Nuclear injection was performed the same as LASER goup.

Chen *et al.* (2004) reported that PIEZO is a superior method to LASER or conventional partial zonal dissecting (PZD) for murine SCNT. However, they did not use PIEZO for nuclear injection after LASER- or PZD-assisted enucleation. For those groups, the aspiration technique was used to break the oolemma before the injection. They performed PIEZO-assisted nuclear injection only for 'PIEZO-assisted' enucleated oocytes. So, their report was about the superiority of PIEZO-assisted nuclear injection, not about that of whole SCNT procedure or zonal opening. Different from the report of Chen *et al.* (2004), we divided experimental groups into LASER- and PIEZO-assisted zona pellucida piercing groups then nuclear injection was performed by PIEZO for both experimental groups (Fig. 1).

Although the data were very limited, the embryos in LASER also showed comparably faster hatching than the embryos in PIEZO (data not shown). It might be from the larger hole on zona pellucida of LASER embryos than that of PIEZO embryos, and this may help hatching the embryos. In human, assisted hatching with acid-Tyrode's solution improves the implantation rate of cryopreserved-thawed embryos (Gabrielsen *et al.*, 2004). However, assisted hatching by laser beams are getting popular in human studies (Tadir and Douglas-Hamilton, 2007). LASER reduced time that took to pierce zona pellucida in the process of enucleation and somatic cell injection, which might have taken longer time with PIEZO. Time and difficulties that took researcher of equivalent skilled for their experiments were decreased with LASER, and this might affect the improvement of embryonic development. In addition, LASER makes the SCNT experiments available for more technicians, which used to be limited to few thoroughly skilled, will activate studies in SCNT research.

In conclusion, laser-assisted zona pellucida piercing improved murine SCNT efficiency and it might by minimizing physical damages from repetitive PIEZO pulses during micromanipulation. Success rate of nuclear injection and embryo development were both improved by using LASER in murine SCNT program. Thus, these data support that LASER can be used for zona pellucida piercing as an alternative to PIEZO.

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