In Vitro Development of Mouse Parthenogenetic Embryos: Effect of Temperature before Oocyte Activation

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ABSTRACT

This study was conducted to establish the optimal temperature condition before oocyte activation in B6D2 F1 mouse. In experiment 1, two embryo culture media (CZB vs KSOM) were evaluated for the development of activated mouse oocytes. Parthenogenetic embryos cultured in KSOM showed better blastocyst development than ones cultured in CZB (56.2% vs 81.0% $p<0.01$). Two-hour of pre-incubation before activation significantly reduced the number of hatched blastocysts in KSOM (22.0% versus 8.8%; $p<0.05$). In experiment 2, recovered oocytes were pre-incubated at different temperature conditions before activation. The experimental groups were divided by 5 as follows. Group A: pre-incubation for 120 min at 37℃, Group B: pre-incubation at 37℃ for 90 min then at 25℃ for 30 min, Group C: pre-incubation at 37℃ for 60 min then at 25℃ for 60 min, Group D: pre-incubation at 37℃ for 30 min then at 25℃ for 90 min, and Group E: pre-incubation at 25℃ for 120 min before activation. Group A (67.6%) and B (66.7%) showed better development to the blastocyst stage than other groups (Group C: 50.0%; Group D: 49.2%; Group E: 33.3%, $p<0.05$). The present study indicates that the temperature before activation affects the development of B6D2 F1 mouse parthenogenetic oocytes and exposure to room temperature should be limited to 30 min when the oocytes are left in HEPES-buffered medium for micromanipulation.

(Key words : Oocyte activation, Parthenogenesis, Temperature, Mouse)

INTRODUCTION

Cloning mammals by somatic cell nuclear transfer was achieved in sheep (Wilmut et al., 1997), cattle (Cibelli et al., 1997), pig (Polejaeva et al., 2000) and rodents (Wakayama et al., 1998; Zhou et al., 2003), although the overall efficiency of generating live offspring from the reconstructed embryos still remains very low. In the mouse somatic cell nuclear transfer (SCNT), donor cell nuclei are usually injected into ooplasms directly using a piezo-actuated micromanipulator instead of membrane fusion between donor cells and the oocytes (Wakayama et al., 1998). Mouse nuclear injection for SCNT is modified from intracytoplasmic sperm injection which is known as ICSI (Kimura and Yanagimachi, 1995). The ICSI operation is currently performed at room temperature (Ogura et al., 2001), although in the original experiments the micromanipulation temperature was 17~18℃ to enhance the survival rate among injected oocytes (Kimura and Yanagimachi, 1995). In the case of hybrid B6D2 F1 (C57BL/6 X DBA/2) oocytes injected with spermatozoa by skilled operators, ICSI efficacy is comparable to that of *in vitro* fertilization (Ogura et al., 2001). It is well known that warming the microscope stage is recommended during enucleation process in SCNT programme because the spindle microtubules will remain well polymerized and form a small clear area, which can be easily distinguished from the surrounding opaque cytoplasm (Nagy et al, 2003). For nuclear injection, however, heated stage cause oocyte lysis. So, the enucleated oocytes should be kept at room temperature during nuclear injection like in ICSI. In the preliminary experiment, we found that artificial activation within 2 h after oocyte recovery supported the developmental competence of rodent parthenotes (Roh et al., 2003). In addition, oocytes exposed at room temperature before activation resulted in lower blastocyst rates than the oocytes kept at 37℃ before activation. Here we showed the optimal temperature condition for B6D2 F1 mouse

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oocytes before activation which is one of the important factors to establish the complete micromanipulation protocols in SCNT programme.

MATERIALS AND METHODS

Reagents and Media

All inorganic and organic compounds were purchased from Sigma Chemical Co.(St Louis, MO) unless otherwise stated. All the media used here were based on CZB and KSOM media (Nagy et al., 2003).

Recovery of Oocytes

Eight-week old B6D2 F1 mice were superovulated by intraperitoneal injections of 7.5 IU equine chorionic gonadotropin (eCG) and 7.5 IU human chorionic gonadotropin (hCG) given 48 h apart. Superovulated females were killed by cervical dislocation at 16 h after hCG injection and the oviducts removed and transferred into a Petri dish containing 2 ml HEPES-buffered CZB medium (HCZB) 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 HCZB before activation.

Activation and *in Vitro* Culture

Recovered oocytes were then immediately exposed to activation medium consisting of 10 mM $SrCl_2$ with 5 μg/ml cytochalasin B in calcium-free CZB for 5 h or cultured in CZB (in an atmosphere of 5% CO_2 in air) or HCZB (in an atmosphere of normal air) for 2 h prior to activation. Following this activation the oocytes were cultured in CZB or KSOM at 37℃ under mineral oil in an atmosphere of 5% CO_2 in air. Detailed experimental design is described belows. Activated and *in vitro* cultured oocytes were then recorded the rates of their development to the blastocyst stage after 4 to 6 day of culture.

Experimental Designs

In experiment 1, two mouse embryo culture media (CZB vs KSOM) were evaluated for the development of parthenogenetically activated mouse oocytes derived from B6D2 F1. The rates of blastocysts were recorded on day 4, 5 and 6 after activation. A part of oocytes were pre-incubated for 2 h in CZB at 37℃ before activation whereas the others activated directly after recovery from oviducts.

In experiment 2, recovered oocytes were pre-incubated at different temperature conditions before parthenogenetic activation. In this experiment, the oocytes were incubated in an atmosphere of normal air (not 5% CO_2 condition) and

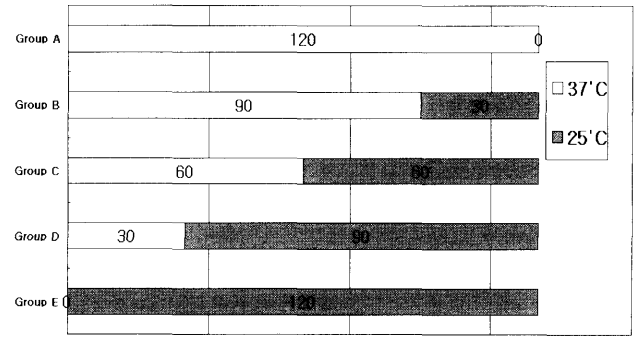

Fig. 1. The scheme of pre-incubation process of B6D2 F1 mouse metaphase II oocytes before parthenogenetic activation. The oocytes were incubated for 120 min in different temperature conditions (Group A: incubation at 37℃ for 120 min; Group B: incubation at 37℃ for 90 min then at 25℃ for 30 min; Group C: incubation at 37℃ for 60 min then at 25℃ for 60 min; Group D: incubation at 37℃ for 30 min then at 25℃ for 60 min; Group E: incubation at 25℃ for 120 min).

HEPES-buffered solution (HCZB) was used as pre-incubation medium. The experimental groups were divided by 5 as follows. Group A: pre-incubation for 120 min in HCZB at 37℃ before activation, Group B: pre-incubation at 37℃ for 90 min then at 25℃ for 30 min, Group C: pre-incubation at 37℃ for 60 min then at 25℃ for 60 min, Group D: pre-incubation at 37℃ for 30 min then at 25℃ for 90 min, and Group E: pre-incubation at 25℃ for 120 min before activation (see Fig. 1 for details).

Statistic Analysis

Differences in the mean percentages of embryonic development among the treatments were analyzed by Chi-Square test.

RESULTS

In experiment 1, mouse parthenogenetic embryos cultured in KSOM showed better development to the blastocyst stage than ones cultured in CZB when the embryos were checked on Day 6 after activation (56.2%, 41/73 *versus* 81.0%, 29/58; $p<0.01$; Fig. 2).

Two-hour of pre-incubation before activation significantly reduced the number of hatched blastocysts in KSOM (22.0%, 9/41 *versus* 8.8%, 6/68; immediate activation *versus* pre-incubation, respectively; $p<0.05$). No hatched blastocyst was found in CZB (Fig. 3).

In experiment 2, Group A (67.6%, 25/37) and B (66.7%, 18/27) showed better development to the blastocyst stage than other groups (Group C: 50.0%, 19/38; Group D: 49.2%, 30/61; Group E: 33.3%, 18/54; $p<0.05$). No difference was found in embryonic hatching rates among all different temperature conditions (Fig. 4).

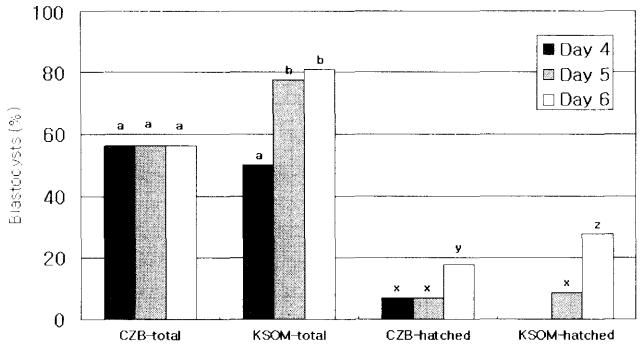

Fig. 2. *In vitro* development of B6/D2 F1 mouse parthenotes in CZB and KSOM media: Comparison of culture media (Day of oocyte recovery and activation: Day 0, $^{ab}p<0.01$, $^{x-z}p<0.05$).

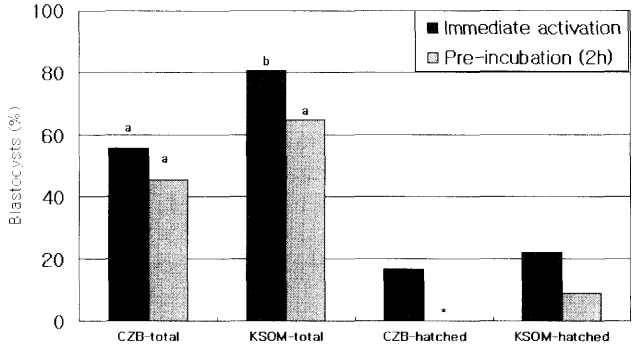

Fig. 3. *In vitro* development of mouse parthenogenetic embryos in CZB and KSOM: Effect of pre-incubation of metaphase II oocytes before activation (Immediate activation: the oocytes were activated immediately after recovery from oviducts, Pre-incubation (2 h): the oocytes were activated 2 h of pre-incubation in CZB medium before activation, $^{ab}p<0.05$, *No hatched blastocyst in this group).

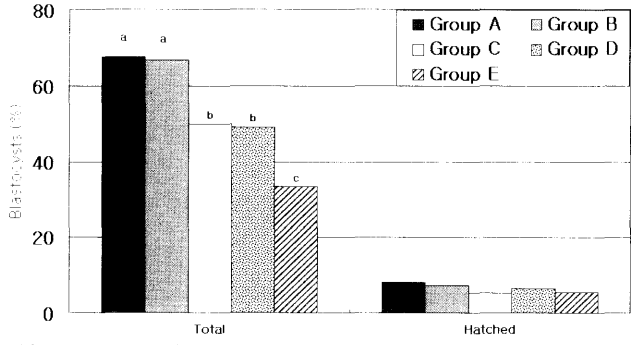

Fig. 4. *In vitro* development of mouse parthenogenetic embryos: Pre-incubation of oocytes before activation in different temperature conditions (Group details: see Fig. 1, $^{ab}p<0.05$).

DISCUSSION

The present study aimed for an improvement of parthenogenetic embryo development in B6D2 F1 mice, which is prerequisite step in SCNT programme. The strain we chose is known as one of the best strain for micromanipulation since MII chromosomes of B6D2 F1 are visible without the use of fluorescence dye and the oocytes of this strain are also tolerant to nuclear injection. First, we tested two kind of culture media (CZB *versus* KSOM) for parthenogenetic embryos of B6D2 F1, which are commonly used for mouse embryo culture. As shown in Fig. 2, over 80% of parthenotes cultured in KSOM developed into blastocysts when checked on Day 6 of culture whereas only 56% of parthenotes developed into blastocysts in CZB. Interestingly, parthenotes in CZB developed faster than those in KSOM. The number of blastocysts were not increased after Day 4 when cultured in CZB, whereas more than 30% of embryos were developed to the blastocyst stage after Day 4 in KSOM. In the next series of experiment, we incubated metaphase II stage (MII) oocytes for 2 h in CZB, then activated and cultured in CZB or KSOM for further development. In the preliminary study, 2 h of pre-incubation in KSOM did not support blastocyst development regardless of the kind of culture media after activation and we chose CZB as pre-incubation medium (data not shown). In SCNT programme, MII oocytes should be exposed to *in vitro* condition at least for 2 ~ 3 h before activation for micromanipulation including enucleation and nuclear injection. Since we found that artificial activation within 2 h after oocyte recovery significantly supported the developmental competence of rodent parthenote (Roh et al., 2003), we limited the duration of pre-incubation to 2 h. Regardless of experimental groups, pre-incubation reduced blastocyst rates in both groups (Fig. 3). No hatched blastocyst was found in CZB whereas 8.8% of hatched blstocysts was obtained in KSOM. Although the embryos in CZB developed faster than in KSOM, actual number of blastocysts were significantly lower in CZB, we chose KSOM as the basic medium for B6D2 F1 parthenogenetic embryo culture. However, as mentioned above, CZB was chosen as pre- incubation medium before activation.

In the final experiment, we tested temperature conditions during the pre-incubation period before activation. Since micromanipulation is usually performed in the air not in CO_2 incubator, we used HEPES-buffered medium (HCZB) for pre-incubation culture in this experiment. Pre-incubation was performed on heated plate not in CO_2 incubator. Somatic cell nuclear transfer programme consist of enucleation, nuclear injection, and activation. It is recommended that enucleation should be performed at body temperature (37℃) to distinguish clear spindle microtubules from surrounding opaque cytoplasm. On the other hand, nuclear injection should be performed at room temperature (25℃) or even at lower temperature (17 ~ 18℃) to avoid oolema lysis after nuclear injection which is commonly occurred in mouse SCNT programme (Nagy et al., 2003; Ogura et al., 2001). Although exposure to room temperature condition is unavoidable in SCNT, it is unclear that how long exposure to room temperature in HEPES-

buffered medium affects oocyte's developmental competence. To test this, we exposed MII oocytes to room temperature from 30 min to 2 h. Total exposure to HEPES-buffered medium before activation was limited to 2 h as described in Fig 1. As summerized in Fig. 4, over 30 min of exposure to room temperature reduced blastocyst developmental rates after activation and *in vitro* culture. So, in SCNT programme, nuclear injection may be completed within 30 min if the injection need to be performed in room temperature.

The results carried out in the present study indicate that temperature before activation affects the development of B6D2 F1 mouse parthenogenetic oocytes and exposure to room temperature should be limited to 30 min when the oocytes are left in HEPES-buffered medium for micromanipulation.

REFERENCES

1. Cibelli JB, Stice SL, Golueke PJ, Kane JJ, Jerry J, Blackwell C, Ponce de Leon FA, Robl JM (1998): Cloned transgenic calves produced from nonquiescent fetal fibroblasts. Science 280:1256-1258.
2. Kimura Y, Yanagimachi R (1995): Intracytoplasmic sperm injection in the mouse. Biol Reprod 52: 709-720.
3. Nagy A, Gertsenstein M, Vintersten K, Behringer R (2003): 'Manipulating the mouse embryo: A laboratory manual 3rd ed.' New York, Cold Spring Harbor Press.
4. Ogura A, Ogonuki N, Takano K, Inoue K (2001): Microinsemination, nuclear transfer, and cytoplasmic transfer: the application of new reproductive engineering techniques to mouse genetics. Mam Genome 12: 803-812.
5. Polejaeva IA, Chen S-H, Vaught TD, Page RL, Mullins J, Ball S, Dai Y, Boone J, Walker S, Ayares DL, Colman A, Campbell KHS (2000): Cloned pigs produced by nuclear transfer from adult somatic cells. Nature 407:86-90.
6. Roh S, Malakooti N, Morrison JR, Trounson AO, Du ZT (2003): Parthenogenetic activation of rat oocytes and their development *in vitro*. Reprod Fertil Dev 15: 135-140.
7. Wakayama T, Perry ACF, Zuccotti M, Johnson KR, Yanagimachi R (1998): Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature 394:369-374.
8. Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KHS (1997): Viable offspring derived from fetal and adult mammalian cell. Nature 385: 810-813.
9. Zhou Q, Renard J-P, Le Friec G, Brochard V, Beaujean N, Cherifi Y, Fraichard A, Cozzi J (2003): Generation of fertile cloned rats by regulating oocyte activation. Science 302:1179.

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