Effects of Different Biopsy Methods on the Development of Preimplantation Mouse Embryos, *in vitro* and *in vivo* : Implication for Preimplantation Genetic Diagnosis

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Abstract

The aim of this study was to investigate the efficacy of two different embryo biopsy techniques, direct aspiration and partial zona dissection (PZD)-push, on subsequent *in vitro* and *in vivo* development of 8-cell stage mouse embryos. It was found that the rates of normal blastocyst formation and hatching blastocysts of direct aspiration, PZD-push, solution control and control embryos were not significantly different (80.8%, 81.6%, 84.5%, 86.7% and 71.9%, 72.3% and 74.6%) respectively. There was, however, a significant reduction in rate of complete hatching blastocysts ($P<0.1$) (72.9% aspiration *versus* 85.2 per cent solution control and 86.4% control) and rate of live-born fetuses (24.2% aspiration *versus* 43.3% solution control and 41.2% control) ($P<0.05$) in the direct aspiration group but no significant difference in the PZD-push group (80.3% of complete hatched blastocysts and 33.8% of live-born fetuses). These findings indicated that embryo biopsy with PZD-push was superior to the direct aspiration method. This mouse embryo biopsy model was useful in advancing development of biopsy technique for human preimplantation genetic diagnosis.

Key word : Embryo Biopsy, Preimplantation Genetic Diagnosis (PGD), Development *in vitro* and *in vivo*

Prenatal diagnosis (PND) for genetic diseases has traditionally involved the screening after a pregnancy is in progress by biochemical or molecular analysis of fetal cells obtained by chorionic villus sampling (CVS) or amniocentesis[1]. However, couples who undergo PND have to face the results of PND; once the fetus is confirmed to be defective, the couples have only one of two choices: termination of pregnancy or having a diseased child.

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Recent advances in the micromanipulation of preimplantation embryo combined with the current techniques available for genetic analysis have made possible a new approach for diagnosis of some inherited diseases prior to implantation of human embryo, i.e. preimplantation genetic diagnosis. This would allow the selection and transfer of an unaffected embryo and establishment of a normal pregnancy[2]. The diagnostic process requires the biopsy of one more cells from each embryo which could be accomplished at very early cleavage stages (such as the two-, four-, and eight- ell stage) or the blastocyst stage. But it is preferable to perform the biopsy at the late cleavage stage (6-to 10-cell) when the loss of one or two blastomeres will cause a lesser reduction in cell number[3]. If embryo biopsy is to be used in a clinical, biopsy techniques must warrant the normal *in vitro* development, *in vivo* implantation and growth of fetus and a good training model with the ability to discriminate harmful effects is a requisite. The use of mouse embryos as a model to perfect the technique of embryo biopsy have been reported, including direct aspiration[4], displacement[5,6], chemical[7] and mechanical[8] zona drilling. However, not all of them have the same potential of success of preimplantation diagnosis[9,10].

The purpose of this study is to compare the efficiency of two mechanical biopsy techniques: direct aspiration and partial zona dissection-push techniques, on the consequent development *in vitro* and the rate of live-fetus birth in the mouse model.

MATERIAL AND METHOD

Animal and embryo collection

Males and females ICR random-bred mice were obtained from the Animal Unit, Department of Medical Science, Ministry of Public Health, and were maintained on a 14L: 10D cycle (light on, 04:00; light off, 18:00). Six-to eight-week old virgin female mice were superovulated by i.p. injection of 5 IU. PMSG (Sigma Chemical Co., St.Louis, MO, U.S.A.) followed by 5 IU. of hCG (Sigma Chemical Co., St.Louis, MO, U.S.A.) 48 hour later. Immediately after hCG injection, females were housed with males of the same strain until the next morning when the mated females were identified by the presence of a copuratory plug in the vagina (day 1 of pregnancy). In order to obtain the 8-cell stage embyros, females were sacrificed in the evening of day 2 (54-56 hours post hCG). Oviducts were excised and the 4-cell embryos were flushed with 0.1-0.2 ml of HEPES-T6 medium supplemented with 10 per cent Fetal Calf Serum (FCS). The embryos were washed 2-3 times in the same medium and then cultured overnight in microdroplets to T6 medium at 37°C with 5 per cent CO_2 in air. In the morning of day 3, all embryos were placed into the biopsy medium, which consisted of a Ca^{2+} - and Mg^{2+} - free buffered salt solution containing 100 mM sucrose and 2 mM ethylenediamine tetraacetic acid (EDTA)[10] and allowed to incubate for 60-90 min. at 37°C to reduce cell-cell interaction. The embryos were transferred to 5 ml of biopsy medium under mineral oil (Sigma Chemical Co., St.Louis, MO, U.S.A.) in 35 mm petri dish (3001, Falcon Plastics, Oxnard, CA) and placed on the microscope stage for micromanipulation. Likewise, control embryos were preincubated in the biopsy medium and placed on a warm stage along with the experimental embryos but without micromanipalation.

Biopsy procedures

All micromanipulations were performed under a Nikon Diaphot inverted phase contrast microscope equipped with three Narishige hydraulic micromanipulators attached to microinjectors (IM-6 Narishige, Tokyo, Japan). Four kinds of micropipettes made from glass capillaries were used in this study : 1) a holding pipette, with outer and inner diameter of approximately 80 and 20 μm, respectively; 2) a sharp-beveled biopsy pipette with 45° beveled angel; 3) a fine- sharpened zona dissecting needle; and 4) a blunted-end biopsy pipette with the diameter of 30-40 μm at tip.

Embryo biopsy was achieved by means of two different techniques: direct aspiration[4,11] (Fig. 1) and modification of the assisted fertilization techniques, partial zona dissection (PZD) of Cohen et al[12], which so-called PZD-push technique (Fig. 2). For both techniques, the embryos were held in place by suction pressure through the holding pipette. During the direct aspiration technique, the tip of a beveled biopsy pipette was pushed through the zona pellucida into the embryo. A single blastomere was removed by aspiration into the column of the biopsy pipette and was then expelled into the surrounding medium. In the PZD-push technique, a small part of zona pellucida was partially dissected

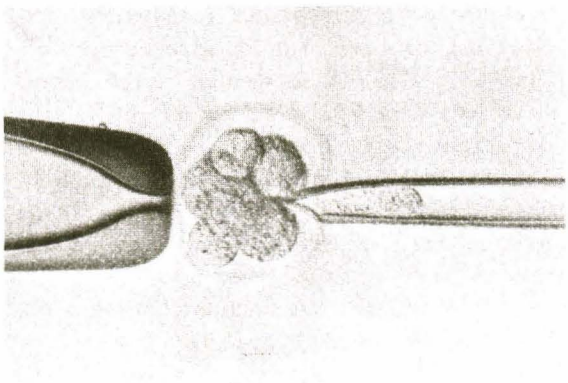

Fig. 1. Direct aspiration precedure for single – blastomere biopsy from an eight-cell mouse embryo held in position by suction on holding pipette. The tip of a beveled biopsy pipette was pushed through the zona pellucida into the embryo and single-blastomere was removed from the embryo by aspiration pressure through the biopsy pipette.

with a sharpened dissecting needle. The embryo with the partial dissected zona was gently squeezed with a blunted-end biopsy pipette until a single blastomere protruded from the embryo. The extruded blastomere was separated from the embryo by sucking it into a biopsy pipette and then expelled into the surrounding medium.

Post-biopsy embryo culture, assessment of development and embryo transfer

All biopsied and control embryos were washed 2-3 times in HEPES-T6 medium before being cultured in microdrops of T6 medium supplemented with 10 per cent FCS and overlaid with pre-equilibrated mineral oil in an atmosphere of 5 per cent CO_2 in air. To determine the effect of the biopsy techniques on subsequent embryo development, two series of experiments were performed.

Experiment I

Biopsied and control embryos were assessed for their further development *in vitro* by means of direct observation under an inverted microsope (400x). The rate of normal blastocyst formation (day 5), *in vitro* hatching (day 6) and complete hatching of embryos (day 7) were compared (Fig. 3).

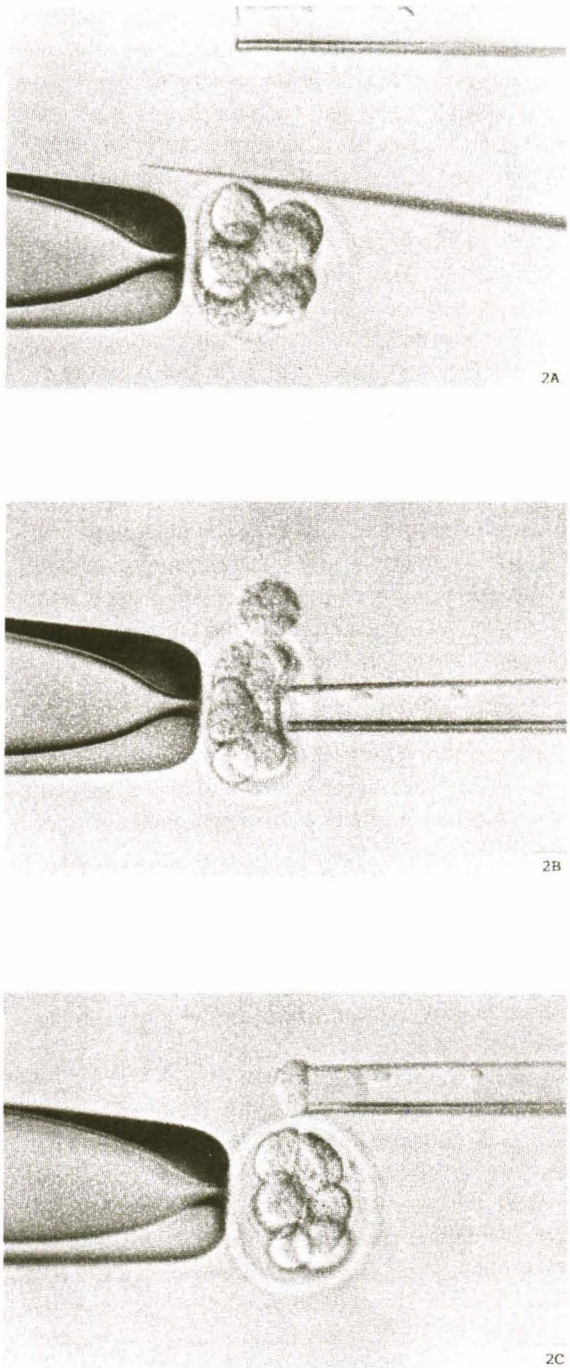

Fig. 2. The PZD-push procedure for single-blastomere biopsy from an eight-cell mouse embryo. (A) A hole is made in the zona pellucida. (B) One blastomere is pushed out of the embryo through the hole in the zona by a biopsy pipette. (C) The expelled blastomere is aspirated into biopsy pipette.

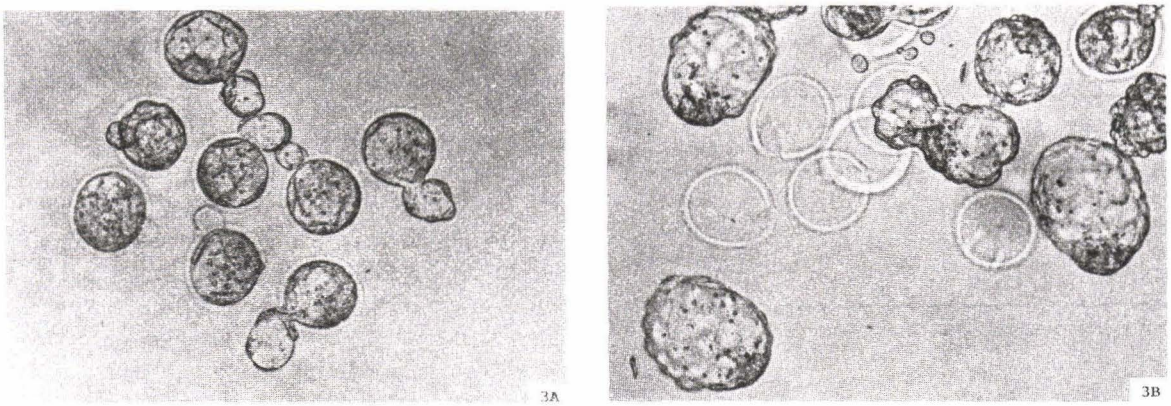

Fig. 3 **A group of biopsied embryos. (A) Hatching embryos, 48 hours after being biopsied. (B) The completly hatched embryos, 72 hours after being biopsied.**

Table 1. ***In vitro*** **development of mouse embryos after biopsy at 8-cell stage as compare to their respective control.**

Treatment	No. of embryos	No. of embryo develop		Rate of complete hatching embryos (D 7)
		Blastocyts (D 5)	Hatching (D 6)	
Control	256	222(86.7)	191(74.6)	165/191(86.4)
Solution control	206	174(84.5)	149(72.3)	127/149(85.2)
Aspiration	271	219(80.8)	195(71.9)	142/195(72.8)[a,b]
PZD-push	294	240(81.6)	229(77.9)	184/229(80.3)

Values in parentheses are per cent
[a]P<0.01, compare to control and solution control
[b]P<0.05, compare to PZD-push

Experiment II

The capacity of biopsied and control embryos to continue post-implantation development was investigated. Forty-eight hours after embryo biopsy, the biopsied embryos at the early blastocyst stage were transferred to the uterine horns of day 3 pseudopregnant recipient mice. The pregnant recipients were housed individually and allowed to deliver at term (19 to 22 days), and the number of living fetuses was recorded in relation to the number transferred in each group.

Statistics

The statistical significance of the results was determined by X^2 analysis.

RESULTS

Development *in vitro*

A total of 1027 eight- cell mouse embryos (256 controls, 206 solution controls, 271 direct aspiration and 294 PZD-push embryos) were evaluated for their capacity to sustain development *in vitro* (Table 1). There were no significant differences in the rate of normal blastocyst formation (day 5) in both biopsied groups when compared with the control and solution control groups (80.8% aspiration and 81.6% PZD-push embryos *versus* 86-4% control and 84.5% solution control). There were also no significant differences in the rate of day 6 blastocyst hatching (including complete and partial hatching) between biopsied and control e mbryos (71.9% aspiration and 77.9% PZD-push *versus* 74.6% control and 72.3% solution control). However, the rate of complete hatching blastocyst (day 7) in the aspiration group (72.8%) was significantly (P<0.01) lower than the control, and solution

Table 2. Rate of live-birth of biopsied mouse embryo after transfer to pseudopregnant recipients.

Treatment	No. of recipients	No. of embryos transferred	No. of live birth fetuses (%)
Control	10	68	28(41.2)
Solution control	10	60	26(43.3)
Aspiration	10	66	16(24.2) [a]
PZD-push	10	68	23(33.8)

[a]P<0.05, compare to control and solution control

control (86.4% and 85.2%, respectively). The PZD-push method apparently improved the rate of total hatching (day 6) (77.9% *vs* 74.6% control and 72.3% solution control) but slightly reduced in the rate of complete hatching blastocyst (80.3% *vs* 86.4% control and 85.2% solution control).

Development *in vitro*

A total of 262 biopsied and control blastocysts after 24 h in culture (68 control, 60 solution control, 66 direct aspiration and 68 PZD-push embryos) were transferred into 40 pesudopregnant recipients and allowed to deliver. The rate of live birth fetuses from the embryos biopsied by direct aspiration method (24.2%) was significantly (P< 0.05) lower that that of the control (41.2%) and solution control (43.3%). No significant difference was found in the rate of live births from the embryos biopsied by PZD-push technique (36.7% *vs* 41.2% control and 43.3% solution control). There was a slight decrease in the live birth fetus rate but not significantly different between direct aspiration (24.2%) and PZD-push (36.7%).

DISCUSSION

We have compared two different embryo biopsy techniques at the 8-cell stage mouse embryos on their subsequent development. The results showed that PZD-push technique involving a partial cutting of zona pellucida and squeezing of single blastomere from the zona slit by pushing against the zona pellucida, appeared to be superior to the direct aspiration technique. Although the normal blastocyst formation between aspiration and PZD-push biopsy techniques were not significantly different, nevertheless, a higher percentage of embryos developed to complete hatching blastocyst and the live birth rate was obtained when the biopsy was performation as the observed end point of *in vitro* development after removal of single blastomeres from cleavage stage embryos[4,8,13]. In this study, no significant differences were found with regard to blastocyst formation (day 5) and hatching stage on day 6 between both biopsy groups and the control groups, but significant differences were evident in the rate of complete hatching on day 7-aged blastocyst in the aspiration group. These findings are in agreement with those of Cui et al[14], thus, the rate of complete hatching from the zona pellucida may reflect the more sensitive end point for discrimination of *in vitro* development. Our results imply that the hatching process may be abnormal following embryo biopsy with aspiration technique. Whether hatching is completed depends on the size of the opening in the zona and the type of procedure. Embryos may become trapped in a characteristic figure of 8. Cohen and Feldberg[15] found that only 16 per cent of PZD embryos migrated through a narrow (<10 μm) hole completely. We found that the opening in the zona made by the aspiration method is a small round hole, whereas, the PZD opening exhibits the appearance of an elongated slit, the same as, but smaller that, in the natural opening in control groups, and most PZD push embryos are more completely hatched from the zona.

One possibility is that failure of hatching per se prevented optimal embryo development *in vivo*. The viability *in vivo* of biopsied embryos is evaluated by transfer of blastocysts of each group into pseudopregnant foster mothers. Our results show impaired postimplantation development of embryos biopsied with the aspiration method. The low live birth rate in the aspiration group may be due to incomplete hatching from zona or failure of implantation in some embryos rather than reduction in cell numbers of developing biopsied blastocyst.

In summary, this study indicates that embryo biopsy in mouse models using PZD-push technique has no apparently deleterious effect on subsequent embryo development. This portends well for advancing development of biopsy technique for human preimplantation genetic diagnosis.

(Received for publication on November 14, 1997)

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การศึกษาเปรียบเทียบผลของวิธีการตัดแยกเซลล์จากตัวอ่อนที่แตกต่างกันต่ออัตราการเจริญของตัวอ่อนในหลอดทดลองและในร่างกาย

พรภิมล ตั้งชัยสิน, วท.ม., กำธร พฤกษานานนท์, พ.บ.**, ทวีศักดิ์ ตีระวัฒนพงศ์, ปร.ด.***, วิทยา ยศยิ่งยวด, ปร.ด.****, ประมวล วีรุตมเสน, พ.บ.***

ได้ทำการศึกษาเปรียบเทียบผลของการตัดแยกเซลล์จากตัวอ่อนหนูเม้าส์ระยะ 8-เซลล์ด้วยวิธีการตัดแยกเซลล์ตัวอ่อน 2 วิธี คือ 1. ดูดเซลล์ตัวอ่อนโดยตรง (direct aspiration) และ 2. ตัดเปลือกหุ้มตัวอ่อนบางส่วนและดันเซลล์ออกมา (partial zona dissection (PZD)-push) ต่ออัตราการเจริญเติบโตของตัวอ่อนในหลอดทดลอง และอัตราการเจริญเป็นฟีตัสมีชีวิต จากผลการศึกษาพบว่า อัตราการเจริญของตัวอ่อนจนถึงระยะบลาสโตซีสและการฟักออกจากเปลือกไข่ของบลาสโตซีสในตัวอ่อนที่ทำการตัดแยกเซลล์ทั้ง 2 วิธีไม่แตกต่างอย่างอย่างมีนัยสำคัญจากกลุ่มควบคุมน้ำยาและกลุ่มควบคุม (ร้อยละ 80.8 ในกลุ่มที่ดูดเซลล์ตัวอ่อนโดยตรง ร้อยละ 81.6 ในกลุ่มที่ตัดเปลือกหุ้มตัวอ่อนบางส่วนและตัดเซลล์ออกมา ร้อยละ 84.5 ในกลุ่มควบคุมน้ำยาและร้อยละ 86.7 ในกลุ่มควบคุม และร้อยละ 71.9 ในกลุ่มที่ดูดเซลล์ตัวอ่อนโดยตรง ร้อยละ 77.9 ในกลุ่มที่ตัดเปลือกหุ้มตัวอ่อนบางส่วนและดันเซลล์ออกมา ร้อยละ 74.6 ในกลุ่มควบคุมน้ำยาและร้อยละ 72.3 ในกลุ่มควบคุม) อย่างไรก็ตาม พบว่า ตัวอ่อนที่ถูกตัดแยกเซลล์โดยวิธีดูดเซลล์ตัวอ่อนโดยตรง มีอัตราการฟักออกจากเปลือกไข่สมบูรณ์ลดลงอย่างมีนัยสำคัญ ($P<0.01$) (ร้อยละ 72.8 ในกลุ่ม เทียบกับร้อยละ 85.2 ในกลุ่มควบคุมน้ำยาและร้อยละ 86.4 ในกลุ่มควบคุม) นอกจากนี้ยังพบว่า อัตราการเจริญเป็นฟีตัสมีชีวิตในกลุ่มน้ำลดลงอย่างมีนัยสำคัญ ($P<0.05$) ด้วยเช่นกัน (ร้อยละ 24.2 ในกลุ่ม เทียบกับร้อยละ 43.3 ในกลุ่มควบคุมน้ำยาและร้อยละ 41.2 ในกลุ่มควบคุม) แต่ไม่พบความแตกต่างนี้ในตัวอ่อนที่ถูกตัดแยกเซลล์ออกโดยวิธีตัดเปลือกหุ้มตัวอ่อนบางส่วนและดันเซลล์ออกมา (ฟักออกจากเปลือกไข่สมบูรณ์ ร้อยละ 80.3 และเจริญเป็นฟีตัสมีชีวิต ร้อยละ 33.8) การศึกษานี้สรุปว่า การตัดแยกเซลล์จากตัวอ่อนโดยวิธีตัดเปลือกหุ้มตัวอ่อนบางส่วนและดันเซลล์ออกมา มีผลกระทบต่อการเจริญต่อไปของตัวอ่อนน้อยกว่าวิธีดูดเซลล์ตัวอ่อนโดยตรง ซึ่งสามารถนำวิธีการตัดแยกเซลล์นี้ไปประยุกต์ใช้กับตัวอ่อนของคนเพื่อการวินิจฉัยโรคทางพันธุกรรมในตัวอ่อนระยะก่อนฝังตัว

คำสำคัญ : การตัดแยกเซลล์ตัวอ่อน, การวินิจฉัยโรคทางพันธุกรรมในตัวอ่อนระยะก่อนฝังตัว, การเจริญเติบโตภายนอกและภายในร่างกาย

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