A Simplified Technique for Embryo Biopsy: Use of the Same Micropipette for Zona Drilling and Blastomere Aspiration

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Purpose: Using different micropipettes for zona drilling and blastomere aspiration for embryo biopsy is prevalent at centers of preimplantation genetic diagnosis. The purpose of our study was to simplify the technique by using only one micropipette.

Methods: In this animal model, ICR mouse embryos at the four-cell stage ($n = 446$) were randomly allocated into two groups: a biopsied group ($n = 224$) for blastomere aspiration and a control group ($n = 222$) without micromanipulation. We used a drilling/biopsy micropipette to drill a hole in the zona by expulsion of acidified Tyrode's solution and to aspirate the blastomere by gentle suction with the same micropipette and pull it out of the zona. One blastomere was biopsied from each embryo.

Results: In all, 222 (99.1%) intact blastomeres were successfully biopsied from 224 embryos. Only two blastomeres were damaged during aspiration. The capacity for blastocyst development (92.4 vs 93.7%) was not different between the two groups, but the percentages of embryos hatching (51.8 vs 18.0%) and hatched (29.9 vs 8.1%) were significantly higher in the biopsied group than in the control group.

Conclusions: This simplified technique of embryo biopsy is safe and highly efficient for obtaining blastomeres for preimplantation genetic diagnosis and may also facilitate hatching of the blastocysts.

KEY WORDS: assisted hatching; blastomere biopsy; zona drilling.

INTRODUCTION

The development of embryo biopsy combined with sensitive molecular genetic techniques provides an

alternative of prenatal diagnosis for pregnancies at high risk of hereditary disease (1–4). Because of its complexity, the clinical application of this method is still limited to a small number of in vitro fertilization (IVF) centers. Therefore, improvements in biopsy techniques and skills for genetic diagnosis are needed. With regard to embryo biopsy, various techniques have been reported in the literature. Some authors use a micropipette to puncture the zona and aspirate the blastomere (5–7). Some authors perform partial zona dissection and then aspirate the blastomere or squeeze the embryo to extrude the blastomere out of the zona (8,9). Other authors drill the zona and then aspirate the blastomere or exert pressure on the zona to push the blastomere out through the hole (10–12).

Among these techniques of embryo biopsy, zona drilling and blastomere aspiration using different micropipettes are the most prevalent at centers of preimplantation genetic diagnosis (13,14). However, it is inconvenient and time-consuming to change the drilling micropipette for the biopsy micropipette. Gibbons *et al.* (15) used three sets of micromanipulators for simultaneous control of the drilling, biopsy, and holding micropipettes to avoid changing micropipettes during biopsy. The purpose of our study was to simplify the blastomere biopsy by using a single micropipette for both zona drilling and blastomere aspiration. We used mouse embryos to examine the feasibility of this technique (7,9,12). Blastocyst formation (6,16) and hatching rate (17) were used as criteria to judge the safety of this technique.

MATERIALS AND METHODS

Preparation of Mouse Embryos

All inbred ICR mice were obtained from the Animal Center of the National Taiwan University Medical Col-

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lege and were originally from Charles River Co., Japan. The mice were maintained on a 12:12-hr light-dark photoperiod at a temperature of 20–25°C. The female ICR mice aged 6–8 weeks were induced to superovulate by intraperitoneal injection of 10 IU of pregnant mare's serum gonadotropin (PMSG; Sigma, St. Louis, MO). Fifty-six hours later, these female mice were injected intraperitoneally with 10 IU of human chorionic gonadotropin (hCG; Pregnyl, Organon, Oss, The Netherlands) to trigger ovulation. Each female mouse was put into the cage of a male to mate individually. Sixteen hours later, mating was determined by the presence of a vaginal plug. Twenty-four hours later, each mouse was anaesthetized by intraperitoneal injection of 2.5 mg Pentothal (Thiopentone sodium, Abbott Australasia Pty. Ltd., Sydney, Australia), and the oviducts were excised and placed into human tubal fluid (HTF) medium (18). The oviducts were opened with hypodermic needles, and the two-cell embryos were collected. The embryos were cultured and checked on the second day for recovery of four-cell embryos. These embryos were randomly allocated to two groups: a biopsied group for blastomere biopsy and a control group without micromanipulation. The culture medium was HTF medium supplemented with 0.5% human serum albumin (HSA; Sigma). The incubation was carried out at 37°C under a humidified atmosphere of 5% CO₂ in air.

Preparation of Micropipettes

Micropipettes were made from thin-walled borosilicated glass tubes (outer diameter, 1 mm) which were pulled by a horizontal puller (Sutter Instrument Company, Novato, CA). For drilling and biopsy with one micropipette, the tapering micropipette was cut with a microforge (Narishige, Tokyo) to an outer diameter of 30 µm, with an inner diameter of 26 µm. The holding micropipette was made with an outer diameter of 80 µm and polished to an inner diameter of 20 µm. For convenience of manipulation on the dish, the micropipettes were bent to an angle of 20°. These micropipettes were then sterilized by dry heat at 160°C for two hr.

Zona Drilling and Blastomere Biopsy

The four-cell embryos allocated for embryo biopsy were incubated in Ca²⁺- and Mg²⁺-free HTF medium supplemented with 0.5% HSA for 2 hr before micromanipulation to decrease blastomeric contact. One droplet (5 µl) of acidified Tyrode's solution (pH 2.4)

was placed in the center of a petri dish (Falcon 3001, Becton Dickinson and Company, Lincoln Park, NJ) and 5 droplets (5 µl/droplet) of 1% Hepes-buffered Ca²⁺- and Mg²⁺-free HTF supplemented with 0.5% HSA was placed on the periphery of it. The droplets were covered by preequilibrated mineral oil (embryo tested; Sigma) to prevent evaporation. Four embryos were transferred into the peripheral droplets (one embryo in each droplet) and the spare droplet was used for cleaning the micropipette during operation. Micromanipulation was performed with the aid of two micromanipulators and microsyringes (Narishige) mounted on an inverted microscope (Olympus, Tokyo) in a modified incubator (19). The drilling/biopsy micropipette was filled and balanced with a small amount of acidified Tyrode's medium. The embryo was held at the 9 o'clock position by gentle suction with a holding micropipette, and the blastomere targeted for biopsy was located at the 3 o'clock position. The zona pellucida was drilled at the 3 o'clock position by expelling acidified Tyrode's solution from the drilling/biopsy micropipette to make a hole 30 µm in diameter (Fig. 1A), and the acid mixed with medium was sucked back into the micropipette immediately once the zona pellucida was opened. After the zona was drilled, the blastomere could usually be seen protruding slightly through the hole. The blastomere was then touched and aspirated gently but firmly by the same micropipette (Fig. 1B). It was slowly pulled out of the zona (Fig. 1C) and released on the margin of the droplet. One blastomere was biopsied from each embryo. The excess medium in the drilling/biopsy micropipette was expelled into the spare droplet, and the micropipette was moved back to the central droplet to be filled again with a small amount of acidified Tyrode's solution for another embryo biopsy. When the biopsies were completed, the embryos were washed and returned to the culture dish.

Evaluation of Subsequent Development and Hatching

The biopsied and control embryos were cultured individually in the microdrops (25 µl) on the petri dish, covered with mineral oil, for a further 4 days. They were observed every day, with emphasis on the development of morula and blastocyst, hatching, hatched, and mode of hatching. Hatching was considered to be initiated when extrusion of a cleaving embryo was observed. The appearance of an empty zona and attachment of the blastocyst to the well indicated that an embryo had hatched.

Fig. 1A. The embryo was held, and the blastomere targeted for biopsy was located at the 3 o'clock position. The zona pellucida was drilled by expelling acidified Tyrode's solution from the drilling/biopsy micropipette to make a hole with a diameter equal to that of the micropipette.

Fig. 1B. After the zona was drilled, the acid mixed with medium was sucked back into the micropipette immediately, and the blastomere was then touched and aspirated gently but firmly by the same micropipette.

Statistical Analysis

The percentages of morula and blastocyst formation, hatching, and hatched embryos were calculated and compared between the two groups. A chi-square test was used for statistical analysis. The difference was considered significant at $P < 0.05$.

RESULTS

In 12 experiments, 541 two-cell mouse embryos were recovered, of which 446 (82.4%) developed to four-cell embryos on the second day. Of these, 224 four-cell embryos were biopsied and 222 embryos were used as controls randomly. In total, 222 (99.1%)

Fig. 1C. The blastomere was slowly pulled out of the zona, then released on the margin of the droplet.

blastomeres were successfully biopsied from 224 embryos, and only 2 blastomeres (0.9%) were damaged by a strong negative pressure during aspiration. The remaining blastomeres were not injured in any of the embryos. Embryo development and embryos initiating hatching and hatched in the biopsied group and the control group are summarized in Table I. The potential for development to morula and blastocyst were not different between the two groups. However, a significantly higher proportion of zona-drilled and biopsied embryos initiated hatching and hatched, compared to the control embryos. The hatching processes in the control embryos exhibited expansion of the blastocyst, thinning of the zona pellucida, extrusion of a cellular projection that penetrated the zona pellucida, and rupture of the zona pellucida. In the zona-drilled embryos, the expanding blastocyst escaped from the hole and the zona pellucida did not become thin.

Table I. Embryo Development and Hatching in the Biopsied and the Control Groups

	Biopsied (n = 224)	Control (n = 222)
Morula	221 (98.7) ^a	218 (98.2)
Blastocyst	207 (92.4)	208 (93.7)
Hatching	116 (51.8)*	40 (18.0)
Hatched	67 (29.9)*	18 (8.1)

^a Values in parentheses are percentages.

* $P < 0.05$ compared to the control group.

DISCUSSION

Mouse embryos are widely used as a model to develop techniques of blastomere biopsy and examine their safety (7,9,12). Both blastocyst formation (6,16) and hatching (17) were advocated as criteria to evaluate the influence of biopsy on the embryos. In our study, a single drilling/biopsy micropipette was used to drill the zona and aspirate the blastomere. The blastomere to be aspirated was located at the 3 o'clock position, where the hole in the zona was made. When the zona was opened, the blastomere usually protruded slightly through the hole. This action might have blocked the flow of acid entering the embryo. The acid was sucked back into the micropipette immediately to reduce the acidity of the environment. An intact blastomere was successfully retrieved in more than 99% of embryos. The biopsied embryos unaffectedly achieved blastocyst formation and hatching. Thus, this simplified technique is safe, effective, and easy for performing blastomere biopsy.

The size of the drilling/biopsy micropipette was designed to be two-thirds the diameter of the blastomere, which allowed making a hole in the zona with a size equal to the diameter of the micropipette but smaller than the blastomere. The blastomere was held firmly and then pulled out without injury. The remaining blastomeres were not lost. The zona-drilled and biopsied embryos had significantly higher percentages of hatching and hatched than those of embryos without micromanipulation. These findings were simi-

lar to those in our previous work regarding partial zona dissection facilitating hatching of mouse embryos in vitro (20). The growing blastocyst may escape from the artificial hole or gap of the zona relatively easily. This may partly explain a higher implantation rate for human embryos after zona drilling in blastomere biopsy for genetic diagnosis (21,22).

Using a single micropipette for both zona drilling and blastomere aspiration is highly efficient and safe for embryo biopsy, and only two sets of micromanipulators are needed. It is more convenient and time-saving than the conventional method, in which micropipettes must be changed during biopsy. It also preserves the integrity of blastomere, and the biopsied material is suitable for preimplantation genetic diagnosis, not only for polymerase chain reaction but also for fluorescence in situ hybridization (FISH). The hole drilled in the zona may facilitate blastocyst hatching. We have also performed this biopsied procedure on excessive human embryos, and the retrieved blastomeres can be successfully fixed and shown in FISH. One-third of the biopsied human embryos reach hatching blastocysts in vitro. The value of this simplified technique of blastomere biopsy in clinical application merits further research.

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