Acta Veterinaria Hungarica 50 (4), pp. 469–479 (2002)

A SIMPLIFIED BIOPSY METHOD FOR PRECOMPACTED MOUSE EMBRYOS: A TECHNICAL REPORT

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(Received February 20, 2002; accepted May 27, 2002)

This article presents a new, simple and rapid embryo biopsy method. The blastomere for genetic analysis can be separated from a precompacted mouse embryo after a partial zona digestion with the use of a holding pipette. For the micromanipulation only two microcapillaries and micromanipulators are needed. The development of the biopsied embryos was studied during *in vitro* culture and *in utero* following embryo transfer. There was no significant difference between the treated and the control groups in the ratio of embryos that developed to the blastocyst stage, although the biopsied embryos were delayed in their development because they contained significantly fewer cells compared to the control ones at the same stage. Although there was no difference in the ratio of implantation, the development of the biopsied embryos *in utero* was also delayed 12–24 hours on the 9th day of pregnancy. No difference in development was visible from the 13th day of pregnancy. Statistically, no differences were found in the developmental ratio (number of developed fetuses/transferred embryos) of the control and treated embryos during gastrulation (9th day of pregnancy), at the beginning of organogenesis (13th day of pregnancy) and before birth (19th day of pregnancy). The embryo biopsy method presented here can be a new and useful tool for preimplantation genetic diagnosis.

Key words: Embryo biopsy, zona digestion, mouse model, preimplantation genetic diagnosis, embryo transfer, embryonic development

In the practice of the preimplantation genetic diagnosis (PGD), a blastomere of a preimplantation stage mouse embryo is obtained for genetic analysis by PCR (Greenlee et al., 1998) or fluorescent *in-situ* hybridisation (FISH; Gimenez et al., 1994). PGD methods in human therapy (Handyside and Delhanty, 1997) and in animal breeding (Bodó et al., 2001; Bredbacka, 2001) were developed in the last decade based on the results of embryo biopsy experiments using

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0236-6290/2002/$ 5.00

the mouse as a model. For the best survival the biopsy is suggested to be performed before compaction (from the eight-cell stage) (Krzyminska et al., 1990). Before micromanipulation, the embryos have to be incubated in Ca^{2+}- and Mg^{2+}-free PBS to reversibly loosen the tight junctions between the blastomeres (Van Blerk et al., 1991; Dumoulin et al., 1998). The blastomeres can then be removed by different methods: by gentle suction with a sharp glass capillary through the slit created mechanically on the zona pellucida (Wilton and Trounson, 1989) or they can be removed with an aspiration capillary through a hole on the zona pellucida created by partial digestion using an acidic medium, e.g. acidic Tyrode's medium (AT) released from a drilling capillary (Gordon and Gang, 1990). The method for mechanical puncture of the zona pellucida requires a sharp aspiration pipette created in several steps during a complicated process using a microforge and a microgrinder. A holder capillary (that holds the embryo during the micromanipulation) is also needed.

For the partial acidic digestion, three capillaries controlled by three different micromanipulators are used: a holder capillary, a drilling capillary, and an aspiration pipette. In human PGD, the partial zona digestion method is used. In 1997 Chen and his colleagues presented a simplified method for the biopsy of mouse embryos that requires only two capillaries. A year later, the same group demonstrated the human application as well (Chen et al., 1998). In that method, the aspiration pipette and the zona-drilling capillary are combined. The weak point of the method is that the capillary has to be refilled from an AT drop after each biopsy. When more than one embryo is biopsied, the process becomes somewhat slow and complicated.

Here we present a similarly simple but faster method developed in the mouse. The mouse model lets us investigate the effect of micromanipulation on the development of the biopsied embryos *in vitro* and *in utero* after embryo transfer. The viability and developmental competence of fetuses on the 9th, 13th, and 19th day of pregnancy were evaluated according to Hogan et al. (1994).

Materials and methods

Embryo recovery and in vitro *culture*

One-month-old C57Bl6/CBA F1 mouse (*Mus musculus*) females were mated after superovulation (Hogan et al., 1994). Plug positive females were killed by cervical dislocation on the second day *p.c.* (post copulation). Embryos were collected from the oviduct and cultured for a day in a humidified atmosphere of 5% CO_2 in air at 37 °C in M16 medium until the 8-cell stage.

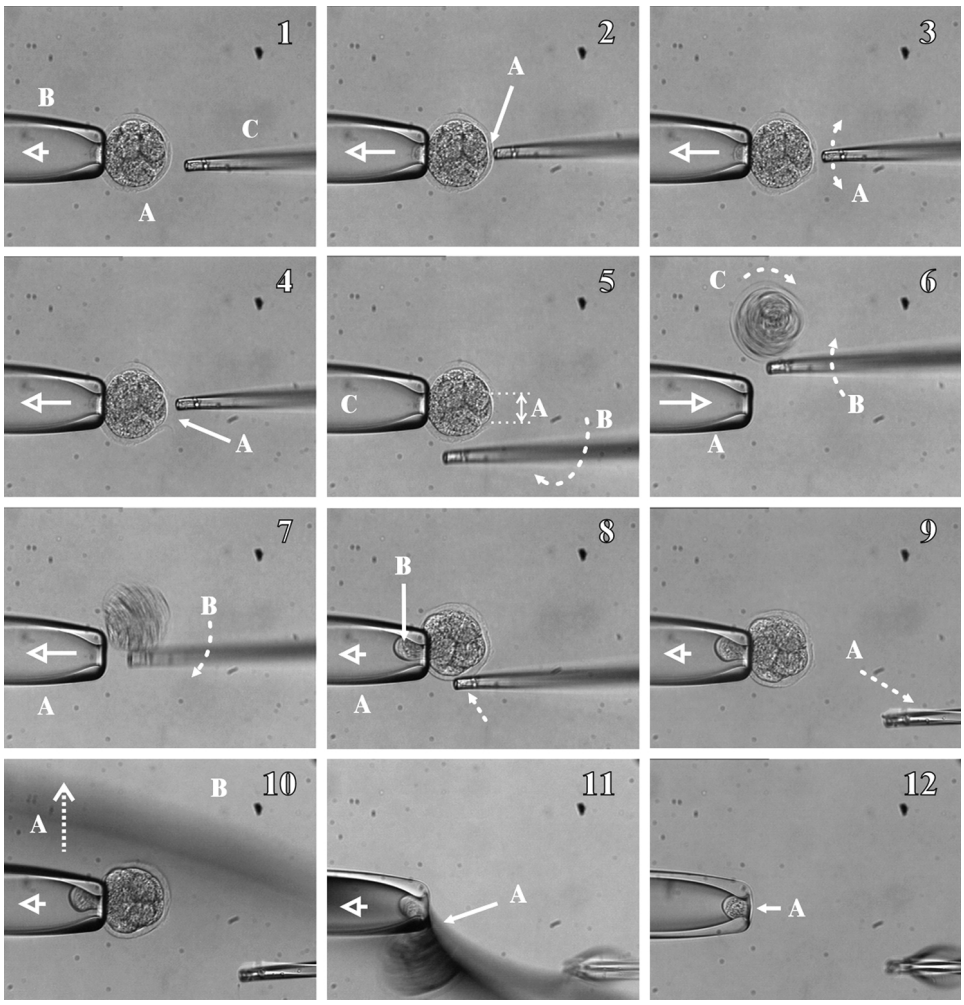
Preparation of microcapillaries

Our simplified method required only two capillaries: a drilling and a holding capillary. They were made of borosilicate glass tubes (GC100-15, Clark Electromedical Instruments, Reading, England) using a horizontal capillary puller (Bachoffer, Reutlingen, Germany). The capillaries differed only in the size of the hole on the tip. For the holding capillary, the pulled glass pipette was broken with a microforge at a point where the outer diameter was 50 μm (Narishige, Tokyo, Japan), and fire polished until the diameter was reduced to half of the original. Then, the capillary was bent in a 20° angle 100 μm from the tip. For the drilling capillary, the pulled glass pipette was broken at 10 μm diameter, and fire polished until the inner diameter reached 6–7 μm. The length of the whole capillary was reduced to 30 mm. The drilling capillary was then filled with AT (pH = 2.4) using a syringe and both pipettes were joined to a pair of Narishige micromanipulators (Narishige Co., Ltd., Japan).

Method of biopsy

Eight- or 16-cell stage precompacted embryos were pre-treated in Ca^{2+}- and Mg^{2+}-free PBS at 37 °C for 20 min to loosen the tight junctions between the cells. For micromanipulation, a special chamber was prepared from a lid of a plastic Petri dish (Greiner, Germany) with a diameter of 35 mm. Each chamber contained five to eight 10 μl droplets of M2 medium covered with paraffin oil (Sigma, USA). One embryo was placed into each pre-warmed droplet. The manipulation was performed on the warmed (37 °C) stage of an inverted microscope (IMT2, Olympus, Japan) using two sets of Narishige micromanipulators at 400 × magnification. The embryo was fixed at 9 o'clock position (Fig. 1A) with the vacuum of the holder capillary (Fig. 1B). The zona-drilling capillary (Fig. 1C) was gently placed on the zona pellucida where the membrane of a blastomere touched the largest area of the inner surface of the zona pellucida (Fig. 2A). The tip of the capillary was moved vertically in a 10 μm track to thin the zona pellucida evenly (Fig. 3A). The flushing AT produced an oval hole (Fig. 4A) with a diameter of 25–30 μm (Fig. 5A). At this moment, the drilling capillary was removed from the embryo (Fig. 5B) and the vacuum of the holder was interrupted to let the embryo loose (Fig. 5C). By means of the medium flushing out of the holding pipette (Fig. 6A) and with the assistance of the drilling capillary (Fig. 6B) the embryo started to rotate (Fig. 6C). Negative pressure was generated again in the holding pipette (Fig. 7A), which along with the aid of the drilling capillary turned the embryo around by 180 degrees (Fig. 7B). A weak negative pressure (Fig. 8A) helped to aspirate a blastomere (Fig. 8B) very gently, in order to maintain its membrane integrity, through the hole of the zona pellucida into the holder capillary. The drilling capillary was removed from the embryo (Fig. 9A) and the microscope stage was moved to position the holder capillary with

the embryo to the edge of the droplet (Fig. 10A). The stage was moved further and the oil layer (Fig. 10B) separated the blastomere from the embryo (Fig. 11A). The separated blastomere (Fig. 12A) was flushed from the holder capillary into the medium droplet.



Figs 1–12. Steps of the micromanipulation. The arrows with dotted line show the direction of movements. The open-headed arrows symbolise the amount of the medium pressure (to left is negative to right is positive) in the holder capillary. Figure legend is included in the text. (Pictures were digitised from a video film recorded in D8 system.)

Following the successful embryo biopsy, the capillaries were relocated into the next droplet without changing the vertical position of the capillaries using only the movement of the microscope stage. While the holder pipette was

kept in fixed position, only the drilling pipette was moved in three dimensions during the whole process.

Embryo culture and embryo transfer

Control and biopsied embryos were cultured in M16 culture medium for a day in a humidified atmosphere of 5% CO_2 in air at 37 °C.

Part of the embryos was used to assess embryonic development on the second day of culture. This was performed by calculating the rate of embryos that developed to the blastocyst stage and determining the total number of nuclei stained with Hoechst 33342 fluorescent dye (Sigma, USA) (Critser and First, 1986). All data were analysed with Student's *t*-test using INSTAT and MS Excel software. The other part of the embryos was transferred into pseudopregnant recipient C57Bl6/CBA F_1 females as described by Hogan et al. (1994).

Viability of transferred embryos

Fetuses were examined at three developmental stages during pregnancy. Pregnant recipients were killed either on the day 9, 13 or 19 of pregnancy. The stage of development and viability of fetuses were evaluated based on the morphology of the embryos being in early somite stage (8.5 day old), at the beginning of the organogenesis (12.5 day old), and of the embryos being in the terminal growing stage, just before birth (18.5 day old), respectively. Data were analysed with Student's *t*-test.

On the 9th day of pregnancy, some of the examined embryos were embedded in JUNG (Leica Instruments GmbH, Germany) tissue freezing medium and frozen in liquid nitrogen. Fifteen μm thin sections were made with a cryostat (MICROM, Heidelberg, Germany) for haematoxylin-eosin double staining. Stained slides were evaluated with a light microscope according to the method of Kaufman (1990).

Five recipient females were let deliver to term. The number and the viability of the pups and their fertility in adulthood were recorded.

Results

Efficiency of micromanipulation

The biopsy was successful in 98% of the cases based on visual evaluation of the membrane integrity of the removed blastomeres. On average, the biopsy was performed in 30 s. The effect of Ca^{2+}-, and Mg^{2+}-free PBS was reversible, the tight connections between the blastomeres were rebuilt in an hour and the embryos began to compact.

In vitro *development of the embryos*

Developmental competence of the biopsied and control embryos is shown in Table 1. The average cell number of the blastocysts in the biopsied group was significantly (15%; $P < 0.05$) less than that in the control group.

Table 1

Developmental competence of biopsied and control embryos *in vitro*

	Number of embryos	Percentage of embryos developed to blastocyst (mean ± SD)	Number of nuclei in blastocysts (mean ± SD)
Control embryos	100	93.7 (± 10.5)	86.5 (± 8.5) [a]
Biopsied embryos	121	92.2 (± 6.7)	59.8 (± 10.1) [b]

[a,b]Values with different superscripts differ significantly ($P < 0.005$)

In vivo *development of the embryos*

Ratios of viable fetuses obtained from killed pregnant recipients at various stages of pregnancy are shown in Table 2. No significant difference was found between the development of biopsied and control embryos. In both groups the number of viable fetuses decreased significantly compared to that of the transferred embryos.

Table 2

Ratios of viable fetuses in different stages of pregnancy

	No. of viable fetuses/No. of transferred embryos (mean % ± SD)					
	8.5 days p.c.		12.5 days p.c.		18.5 days p.c.	
Control embryos	54/70	(78.8 ± 24.2)[a]	49/68	(72.1 ± 22.2)[a]	27/49	(55.1 ± 25)[b]
Biopsied embryos	52/68	(76.5 ± 21.4)[a]	49/73	(67.1 ± 19.7)[a]	35/60	(51.2 ± 22)[b]

[a,b]Values with different superscripts differ significantly ($P < 0.05$)

The growth and development of fetuses developed from the biopsied embryos (Fig. 13) was delayed by 12 or 24 h on the 9th day of pregnancy compared to the controls (Fig. 14). However, no developmental differences were detected between the fetuses on the 13th or the 19th day of pregnancy.

Histological examinations were carried out on 8.5-day-old fetuses. According to the results of the haematoxylin-eosin staining, the embryonic layers were intact and no developmental deformities were detected in either group.

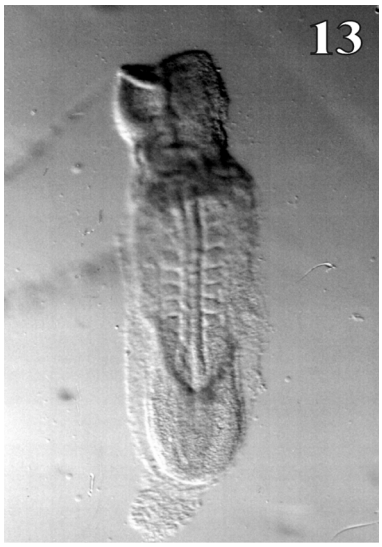


Fig. 13. Biopsied embryo on the 9th day of pregnancy

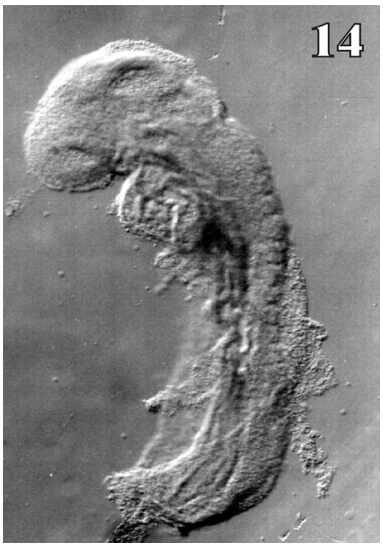


Fig. 14. Control embryo on the 9th day of pregnancy

Characterisation of the offspring

The recipient females gave birth to healthy offspring (27 pups from 44 transferred biopsied embryos) on the 19th day of their pregnancy. These animals reached adulthood and were mated in consecutive brother-sister mating. The F_2 females gave birth to healthy offspring with 8–9 pups/litter, that was not different from the natural ratio in $F_2 \times F_2$ mating. Fertility of the adult animals born from biopsied embryos was normal.

Discussion

A new embryo biopsy method was demonstrated and its effects on the *in vitro* and *in vivo* embryonic development were assessed.

Remarks on micromanipulation

The results indicate that a quick and easy method was developed. It requires only two microcapillaries that are easy to make without the use of a microgrinder. Two aspects had to be taken into consideration during the development of the method. First, the embryos had to survive the micromanipulation, and second the blastomeres obtained had to be intact for the analysis. The use of Ca^{2+}- and Mg^{2+}-free PBS is indispensable, especially in the case of the compact embryos because the vacuum of the holder may injure the cells if the intercellular connections are tight. The effect of the Ca^{2+}- and Mg^{2+}-free PBS is reversible

(Santalo et al., 1996) and considering the duration of the biopsy, the optimal number of embryos in the micromanipulation dish is five to eight. This ensures that the intercellular connections are still loose enough to remove the blastomere safely and easily even in the last embryo. In 8-cell embryos, the blastomere can be removed from the embryo by the vacuum of the holder or with a careful flick of the drilling capillary from the position of 6 o'clock to 12 o'clock. In compacting (10–16 cell) embryos, the integrity of the blastomere can be ensured only if the borderline between the medium drop and the oil is used to remove the cell. In case of the last embryo in the dish, it is also more practical to use this latter method.

Several capillaries should be made prior to biopsy and if a capillary becomes contaminated with debris from a damaged blastomere, it has to be replaced with a new one to prevent contamination with DNA.

Remarks on the in vitro *development of the biopsied embryos*

No difference was found in the *in vitro* development (in terms of hatching from the zona pellucida) between the biopsied and the control embryos. However, the biopsied embryos had significantly less nuclei that can partly be explained with the removal of a blastomere and thus decreasing the number of cleaving cells in the third and fourth cell cycle. The micromanipulation and the process of recompaction can also set back the rate of cell division.

The occurrence of hatching does not necessarily refer to hatching as a developmental stage. Two embryos hatching at the same time does not mean that they are at the same developmental stage. This phenomenon was first reported by Malter and Cohen (1989). The expansion of the blastocysts that normally comes with a significant increase in diameter and the thinning and slitting of the zona pellucida is only partial in biopsied embryos. A part of the embryo expands through the hole made on the zona pellucida during expansion, although the diameter and the thickness of the zona do not change noticeably. Hatched biopsied embryos are set back in development by half a day having nucleus numbers similar to those found in late expanding blastocysts.

Remarks on the development of embryos in utero

The delay in the development of the biopsied embryos observed *in vitro* was also studied *in vivo* following embryo transfer.

It was assumed that the difference in the development of embryos from different treatment groups could be observed during pregnancy. The turning of the control embryo can be observed on the 9th day of the pregnancy, when the gastrulating embryo is in the early somite stage – Thelier stage 13 (Kaufman, 1992). However, the size and development of embryos that underwent biopsy are decreased and they can be classified as Thelier stage 12. Despite the delayed de-

velopment, no developmental abnormalities were found in histological examinations. There was no difference in organogenesis nor in size among the 12.5-day-old fetuses. All of them were found to be in the Thelier stage 20. On the 19th day of pregnancy, immediately before birth, no difference was found between fetuses. In contrast to the observation of Hsu et al. (2000), we found that the micromanipulation performed on preimplantation embryos adversely affected the development of embryos during implantation and early pregnancy. This delay can probably be attributed to recompaction, and the absence of blastocyst expansion before early hatching. Our results suggest that the fetuses are able to offset this delay during organogenesis by the 13th day of pregnancy and develop normally. Although there are reports demonstrating the decrease in implantation of biopsied embryos (Wilton and Trounson, 1989), the only difference found in the present study was in the early fetal development of the biopsied and control embryos. The compensation observed in the early phase of organogenesis requires further studies.

Characterisation of progeny

The micromanipulated embryos developed into healthy, fertile adults able to breed normally. Based on blood analysis (Cui et al., 1993), and histopathological analysis (Cui et al., 1994), it can be assumed that the micromanipulation does not cause postnatal aberration. No difference was observed in the fertility, gestation length and number of pups between the adults born from biopsied and control embryos.

It can be concluded that this micromanipulation method is quick and simple, it does not decrease the viability of the precompacted embryos and leaves the membrane of the removed blastomere intact. The production of the micropipettes requires only a microforge. For micromanipulation, a micromanipulator is needed only that moves in a range of millimetres in 3D to control the drilling capillary. The holder is fixed at the level of the micromanipulation. This is an advantageous feature in the practice, as only a simple, inexpensive control unit is needed for the holder if the inverted microscope has a movable stage. Unlike the method of Chen et al. (1997), the capillary does not need to be refilled with AT solution after each biopsy, thus the work can be done much faster.

The *in vitro* development of the manipulated embryos does not differ from that of the controls, however, the difference observed in the nucleus number indicates that there is a setback in the developmental potential because of the absence of the removed blastomere and because of early hatching. However, it was found that this was compensated during organogenesis. The pups born had normal phenotype.

Having a short gestation period, mice are ideal to study the effects of micromanipulation on preimplantational embryos. It is probable that the manipulation would have similar effects on embryos of domestic animals or humans.

The study of the physiological effects of the method in the mouse is a prerequisite to its introduction into practice. The use of the mouse as a model animal may greatly decrease the uncertainty originating from the environmental effects that may bias the correct evaluation of the results.

Acknowledgements

The authors wish to express their gratitude to Julianna Kobolák and Mária Gróf for their help. We thank also Zoltán Macháty, PhD, for reviewing and correcting the style of the manuscript. This project was financed from grant FKFP – 0469/1997 of the Hungarian Ministry of Agriculture and Rural Development.

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