**SJBS (B),2003,8: 55-59**

# IMPROVEMENT OF MOUSE EGG MICRODISSECTION

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## ABSTRACT

In this study three experiments were designed to simplify the microdissection of the mouse egg (oocyte and pronuclear stage embryo). In Experiment 1: 200 zona-free oocytes were pipetted gently using a micropipette with an internal diameter of 20- 30 µm. This treatment resulted in deforming of eggs into cylinderical rods. Then the deformed oocytes were dissected manually under a dissecting microscope by a fine glass needle. The rate of dissection success was 95% and at least 70 eggs can be dissected in an hour. In Experiment 2: Pronuclear stage embryos were dissected as in Experiment 1 into karyoplasts and cytoplasts. The karyoplsts were cultured *in vitro* to test their developmental ability. The development of karyoplasts, to balstocyst stage was very low (20%). In Experiment 3: The cytoplasts obtained in the above experiments were aggregated with 2-cell stage blastomeres in Whitten medium containing 10 µg/ ml phytohemagglutinin. The aggregated couplets were fused in phosphate buffered saline (PBS) or 0.3 M mannitol to test their tolerance to fusion media. The produced reconstituted embryos were cultured *in vitro* to evaluate their development. The fusion rates were significantly higher in PBS than in 0.3 M mannitol ($p<0.05$). Furthermore, the development of reconstituted embryos obtained by fusion in PBS was higher than those obtained by fusion in mannitol.

This study provides a simple, rapid and easy manual microdissection of mouse eggs into cytoplasts and karyoplasts with very high rate of success. Furthermore, the cytoplasts and karyoplasts obtained can be used in embryo reconstitution, embryo cloning and nucleocytoplasmic interactions studies.

Key words: Cloning, fusion media, microdissection, mouse egg, reconstitution.

## INTRODUCTION

The enucleation procedure is now one of the most time consuming step in embryo cloning (Steven and First, 1993). Some researchers described some efficient techniques for enucleation (McGrath and Solter, 1983; Tsunoda *et al*., 1986). But all these kinds are expensive, time consuming and somewhat difficult to master. Enucleation of zona-free mouse eggs into cytoplast and karyoplast had been done manually by microdissection of the mouse egg (Tarkowski, 1977). However, it is not easy to dissect the mouse egg when it is round perfectly. A microdissection technique was also described by Barton and Surani, (1983) and Willadsen, (1986), however, it needs special equipment and the number of the dissected eggs per hour is 20 -30. Enucleation of zona-free hamster and mouse oocytes was also done by centrifugation through percoll gradient (Yanagimachi and Yang, 1990). Laser rays have been used to ablate early embryo blastomere (Steven and First, 1993). A novel approach to enucleate oocytes by ultraviolet rays was described by *Tsunoda et al.* (1988). All basic research on nucleocytoplasmic interaction and embryo cloning was done using one of the above mentioned techniques. The use of the manual dissection as a mean for embryo cloning and nucleocytoplasmic interaction studies is not popular (Tarkowski, 1977; Taniguchi and Kanagawa, 1992).

The aim of this study was to develop a simple, rapid and easy manual microdissection technique to obtain inexpensive cytoplasts and karyoplasts, that can be used in nucleocytoplasmic interactions studies, reconstitution of embryos and embryo cloning. Moreover, tolerance of the produced cytoplasts to fusion media was investigated.

## MATERIALS AND METHODS

Collection of Oocytes and Embryos

ICR and F1 (C57BL/6 x CBA) female mice were superovulated with 5 IU of pregnant mare serum gonadotropin (eCG, Serotropin, Teikoku Zoki, Japan), followed by 5 IU of human chorionic gonadotropin (hCG, Gonatropin Teikoku Zoki Japan) 48 hours later. Mature oocytes were obtained from F1 females scarified 17 - 19 hr post hCG injection. For collection of pronuclear stage embryos, females were placed with males of proven fertility after hCG injection. F1males, which have vaginal plugs 15 hrs after hCG injection, were scarified 5 - 7 hrs later. Ampullae of the oviducts were dissected and placed in sterilized tissue culture dishes (35 x 10 mm, Nunc, Roskilde, Denmark) containing Dulbecco, s phosphate buffer saline (PBS) supplemented with 10% fetal calf serum(FCS), (Gibco, NY, U.S.A). The oocytes were collected by piercing the oviducts. Late 2-cell stage embryos were collected from ICR females, mated with F1 males, at 44 - 46 hr post hCG injection. For collection of late 2-cells embryos the oviducts were flushed with Whitten,s medium supplemented with 0.1 mM ethylenediamine tetraacetic acid (Whitten, 1971). The cumulus cells were removed from the eggs by treatment with 300 IU /ml hyaluronidase (Type I-S Sigma, U.S.A) in PBS. Zonae were removed from oocytes and embryos by pipetting after exposure to 0.5% pronase (Actinase; Kaken Pharmaceutical Co., Tokyo, Japan) as described by Mintz (1962). Blastomeres of the 2-cell embryos were isolated after culture in calcium free Whitten,s medium (Elsheikh *et al*.,1997 a) for 15-30 min.

Microdissection

This is performed manually under a dissecting microscope (SMT-2T time 63, Nikon Co. Ltd. Tokyo Japan) on 1% agar (Gibco) in 0.85% PBS (Elsheikh *et al*. 1997 b). Zona-free eggs were transferred to the manipulation medium (agar covered with PBS + 10 % FCS) in-groups of 5-10 eggs at a time. They were then pipetted gently using a micropipette with an internal diameter of 20- 30 µm. This treatment resulted in deformation of eggs into cylinderical rods. The rods were

then dissected manually into karyoplasts and cytoplasts by a fine glass needle of 5 µm and curved to 30 degree on microforge. The position of the polar body was used as indicator for the presumed localization of the oocyytes chromosomes to dissect them into cytoplast and karyoplasts.

Aggregation of 2-cell stage blastomeres with cytoplasts into couplets

The isolated blastomeres were aggregated with the produced cytoplasts (Fig. 1) in Whitten medium containing 10 µg/ ml phytohemagglutinin (PHA, Difco laboratories, NY, U.S.A) as described elsewhere ((Mintz *et al.,* 1973; Elsheikh et al., 1997 a) .

Electrofusion of the couplets

The aggregated couplets were pipetted into the fusion chamber about 5 pairs at a time. The fusion chamber consisted of 2 stainless steel plate electrodes (25.0 x 15.0 x 0.5 mm) glued onto a glass slide 0.5 mm apart (Elsheikh *et al*., 1995). About 300µl from the fusion solution was placed between the electrodes. The fusion solution was PBS + 10% FCS or 0.3 M mannitol to which 0.05 mM of $CaCl_2$ 2H2O, 0.1 mM Mg $SO_4$ and 0.05 mg/ml bovine serum albumin(BSA) were added. An electric cell fusion instrument Model LF 100, Life.Tec. generated the electrofusion pulses. Co. Ltd. Tokyo, Japan). The couplets were manually oriented so that the fusion plane was parallel to the electrodes. They were then electrofused by 2 direct currents pulses (150 v/mm, 70 µsec duration and one second interval), proceeded by an alternative current of 5 v (500 KHS) for five second. (McGrath and Solter, 1983; Kubiak and Tarkowski, 1985; Henery and Kaufman 1992; Elsheikh *et al., 1995*) The fused couplets (Fig. 1) were activated by 7% ethanol in PBS for 5 min (Elsheikh *et al*., 1997 b) and then cultured in Whitten's medium for 96 hr at 37o C in an atmosphere of 5% CO2 in air. Development of the reconstituted embryos was examined under an inverted microscope.

Experimental design

Experiment 1: This experiment was carried out to study the effect of manipulation on the healing of the cytoplasmic membrane and survival rate of dissected oocytes. Zona-free oocytes with polar bodies were bisected into karyoplasts and cytoplasts. The karyoplasts and cytoplasts were then cultured in Whitten,s medium for 3 hr at 37oC in atmosphere of 5% CO2 in air. The healing of the cytoplasmic membrane was observed under an inverted microscope (Diphot, Nikon) to determine the survival rate of the fragments.

Experiment 2: This experiment was done to examine the effect of extreme manipulation on the developmental rate of karyoplasts obtained from bisected pronuclear stage embryos. Zona-free pronuclear stage embryos were dissected into cytoplasts and karyoplasts. The karyoplasts were cultured in Whitten,s medium for 96 hr at 37oC in atmosphere of 5% CO2 in air and their development to blastocyst was observed.

Experiment 3: This experiment was carried out to compare the effects of fusion media on fusion rates of aggregated couplets and the subsequent development of reconstituted embryos (fused couplets). The cytoplasts derived from the previous experiments were aggregated and fused with blastomeres from the 2-cell embryos as described above.

Statistical analysis

The results were statistically evaluated with Chi-square. Differences at a probability of $p<0.05$ were considered to be statistically significant.

## RESULTS

Experiment 1

Out of 200 dissected oocytes, 190 oocytes survived and tolerated the micromanipulation procedure. Immediately after dissection the cytoplasmic membrane healed and both of the oocyte fragments became round (Fig. 2).

Experiment 2

After dissection of pronuclear stage embryos all the cytoplasts and karyoplasts rounded up and become intact immediately (Fig. 3). However, 5.3% of the karyoplasts cultured developed to the blastocyst stage was 5.3% (Table 1).

Experiment 3

The fusion rate of the aggregated couplets in PBS + 10% FCS was high compared with those fused in mannitol ($p<0.05$). Furthermore, the developmental rates of reconstituted embryos obtained by fusion in PBS were also high as compared with those obtained by fusion in mannitol solution.

## DISCUSSION

For commercial use of nuclear transplantation techniques and embryo cloning a large number of recipients cytoplasm of good quality and easy to produce is needed (Steven and First,

1993). The microdissection technique described here was combined from the one described by Tarkowski (1977) and that described by Barton and Surani (1983). By this technique we can spare a lot of time to obtain cytoplasts for embryo reconstitution with very high survival rate with less effort and simple equipment. At least 70 oocytes can be dissected by this technique in an hour. As mentioned before microdissection can be done as described by Tarkowski (1977), however it is not easy to dissect the oocyte without deforming it into a cylinderical rod. Moreover the needle need to be changed frequently. The technique described By Barton and Surani (1983) although it is efficient but it needs special equipment and only about 20-30 oocytes can be dissected in an hour. The technique described here allows the production of large number of cytoplasts. As the distortion of the eggs has no detectable detrimental effects (Barton and Surani, 1983), this step can be carried manually and quickly.

From Experiment 1, a high rate of oocyte fragments survival up to 95% can be attained by this technique. The developing rate of bisected embryos to the blastocyst stage was 20%. This is mainly due to the fact that, the karyoplasts cultured include different kinds of karyoplasts (androgenetic karyoplasts, gynogenetic karyoplasts and karyoplasts with less than 50% cytoplasm). These kinds of karyoplasts showed poor development according to Barton and Surani, 1983). From Experiment 3, Exposing the cytoplasmic membrane to extreme manipulation procedures during rods making and bisection may have been sufficient to cause lysis once the couplets were placed into mannitol solution specially the cytoplasts, due to the ionic difference between the culture medium and mannitol solution. This increased rate of lysis resulted in reduction of the fusion rate. However when we used PBS+ 10% FCS as fusion medium the rate of fusion increased due to the decrease in the rate of lysis. The cytoplasts obtained by this method can be used in embryo reconstitution utilizing electrofusion techniques in electrolyte fusion medium ( Richord and White, 1992; Elsheikh *et al*., 1995).

The only drawback of this method is that, the cytoplasts produced are zona free. With progress in embryo aggregation, embryo fusion, microencapsulation (artificial zona) (Cosby and Dukelow, 1990; Elsheikh *et al*. 1997 b) or techniques utilizing empty zona (Bondioli *et al*. 1990), this technique will serve as an efficient tool for embryo cloning.


## ACKNOWLEDGEMENTS

We would like to thank Dr. A. Pinyopummin and Dr. H. Tanaka for their invaluable help. We are also in debt to our colleagues for their assistance in the laboratory works.


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Table 1. Development of dissected one cell stage mouse embryos.

| Trails | No. dissected | No. degenerated | Fragments* cultured | No. of fragments developed to | | |
|---|---|---|---|---|---|---|
| | | | | 2-cell | 4-cell | Blastocyst |
| 1 | 20 | 3 | 17 K | 10 | 3 | 1 |
| | | | 17 C | 0 | 0 | 0 |
| 2 | 20 | 6 | 14 K | 8 | 1 | 0 |
| | | | 14 C | 0 | 0 | 0 |
| 3 | 30 | 4 | 26 K | 15 | 2 | 2 |
| | | | 26 C | 0 | 0 | 0 |

* K: karyoplast, C: cytoplasts.

Table 2. The effects of electrofusion treatment on the fusion and development of aggregated mouse couplets.

| Fusion medium | No. treated | No.(%) fused | No. not fused | No. Deg. | No. (%) of embryos developed to* | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | 2-cell | 4-cell | Morulae | Blastocysts |
| Mannitol | 40 | 23 (57.5)a | 3 | 20 | 8 (34.9)[a] | 8 (34.9)[a] | 2 (8.7)[a] | 0 (0)[a] |
| PBS + FCS | 69 | 50 (72.5)b | 11 | 8 | 22 (44)[b] | 14 (28)[b] | 14 (28)[b] | 10 (20)[b] |

* Percentages are based on the number of embryos fused.

PBS+ FCS: Phosphate buffered saline plus 10% fetal calf serum.

[ab] Values with different superscripts in the same column differ significantly (p0.<0.05).

Fig. 1. A) Aggregated Blastomere (Small arrowhead) and cytoplast (large arrowhead). 5 400

Fig.1 B) The aggregated blastomere and cytoplast in the process of fusion. 5 400

Fig.2. A) Zona-free oocytes pipetted with a pipette of 20-30 μm internal diameter into cylindrical rods. B) Cytoplasts obtained after manual dissection. 5 400

Fig.3. A) Zona-free pronuclear stage embryo pipetted with a pipette of 20-30 μm internal diameter into cylindrical rods. 5 400

Fig. 3. B) Karyoplast (Large arrowhead) and cytoplast (small arrowhead) immediately after manual dissection. 5 400

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