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Optimal Method of Mouse Blastomere Biopsy: *In vitro* Developmental Potential of the Biopsied Embryo to Blastocyst Stage after Aspirated Eight-Cell Mouse Embryos

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Abstract: The researchers investigated the effect of blastocyst development and quality of blastomere aspiration techniques on eight-cell mouse embryos. The results clearly indicate that the *in vitro* development of biopsied mouse embryos depended on the suitable aspiration method. This method related to pre-implantation genetic diagnosis for the genesis of animal models for animal disorders from embryos. In this study, female CB6 F1(Balb/cXC57bl/j) hybrid mice were superovulated with hormones and superovulated females were sacrified approximately 68 h after hCG administration. About eight-cell embryos were recovered from oviducts of sacrified mouse in M2 medium. Before biopsy, all embryos were incubated to decrease cell to cell contacts to microdrops of Ca^{2+}/Mg^{2+} free QAM HTF (3 mg mL^{-1} BSA Fraction V) with HEPES for 90 min at 37°C. A single blastomere of eight-cell embryos were aspirated by the aspiration pippetes under inverted microscope (4X). After biyopsy, embryos were cultured in SAGE medium supplemented with 5% CO_2, 5% O_2 and 90% air at 37°C for up to expanded blastocyst stage. After culture, 267 biopsied embryos out of 234 (88.32±7.5%) expanded blastocysts developed from the biopsy group and total cell number mean 67.8±17.2% were recorded in the experiment group and 126 non-biopsied embryos out of 118 (94.4±7.78%) expanded blastocysts developed from control embryos and total cell number mean 70±15.4% was recorded in the control group. In the present study, there was no difference in blastocysts developmental rates at post biopsy group and control group ($p = 0.05$). In conclusion, the biopsy the method described here is an optimal method of blastomere aspiration on *in vivo* mouse embryos.

Key words: Blastomere, embryo biopsy, mouse embryos, micromanipulation, hormones, biopsy group

INTRODUCTION

Embryo biopsy and single cell genetic analysis now make it possible to screen human and animal embryos at preimplantation stages of development. In the human, Pre-implantation Genetic Diagnosis (PGD) techniques are being used to screen the embryos for diagnosing genetic defects causing a number of inherited diseases and other genetic conditions (Handyside and Delhanty, 1997). Pre-implantation embryo biopsy found its first clinical application in human prenatal diagnosis and human and animal sex determination of pre-implantation embryos derived from IVF (Handyside *et al*., 1990). Biopsy of pre-implantation embryos may offer new way for genetic diagnosis. Blastomere aspiration of pre-implantation embryos is an important technique which has been widely used in mammalian reproductive bio-technologic genetic pre-diagnosis and sex determination of embryos also animal models for animal and human disorders from embryo. Mammalian embryo biopsy first was applied in the mouse (Tarkowski, 1959; Tarkowski and Wroblewska, 1967). Mouse embryos were biopsy two different stage biopsy by cutting the trophectoderm of mouse blastocysts and aspiration of blastomeres from the eight-cell stage embryo (Monk and Handyside, 1988). Blastomere aspiration of preimplantation mouse embryos is obtained for genetic diagnosis by PCR. A eight-cell stage is most suitable than 4 cell biopsy which mouse embryos biopsied at diffirent pre-implantation stage of development (Krzyminska *et al*., 1990). Also, this technique used in human therapy (Handyside *et al*., 1997) and used serial biopsied on diferent stage embryos for blastocysts development (Illmensee *et al*., 2006). Blastomere aspiration technique is very useful for farm animals, in sheep, 36% of embryos biopsy as 2 and 4 cell embryos developed to term following transfer to recipient females (Willadsen, 1980). Sexing of mouse pre-implantation embryos was done by detection of PCR (Kunieda *et al*., 1992). The nervous system could be sensitive to blastomere biopsy procedures and

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indicated an increased relative risk of neuro-degenerative disorders in the offspring generated following blastomere biopsy (Yu *et al*., 2009). Embryo biopsy has potential clinical relevance in that it demonstrates that chromosomally defective embryos can be accurately before implantation (Kola and Wilton, 1991). In the present study, the researcher examined in *in vitro* development potential of biopsied eight-cell mouse embryos from which one blastomere were aspirated by micro-manipulation.

MATERIALS AND METHODS

Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated. All animal care and use procedures were in accordance with the institutional guide for the care and use of laboratory animals and TUBITAK animal care and use ethic committee.

Superovulation and embryo recovery: CB6F1 (C57BL/6 X BALB/c-F1) hybrid mice were maintained under a 14 h light/10 h dark cycle. Females (5-6 weeks of age) were superovulated by intraperitoneal administration of 10 IU pregnant mare serum gonadotropin (PMSG; cat. No. G-4877) and 48 h later by 10 IU human Chorionic Gonadotropin (hCG) hormones (Pregnyl; Organon, Whitehouse station, NJ, USA) (Bagis *et al*., 2009). The females were placed with males for mating and examined on the following morning (day 0) for the presence of vaginal plug. Approximately, 66-68 h post hCG oviducts and cornu uteri were excised and eight-cell embryos were flushed in M2 medium then transferred into fresh M2 medium (Krzyminska *et al*., 1990). Finally, all embryos were washed three times in M2 medium and eight-cell embryos were selected and used for the experiments. Before embryo biopsy, all embryos were preincubated for 60-90 min to decrease cell to cell contacts to 10 μL microdrops of Ca^{2+}/Mg^{2+} free bicarbonatte buffered QAM HTF (3 mg mL^{-1} BSA Fraction V.) with HEPES (Wilton and Trounson, 1989; Santalo *et al*., 1996).

Micromanipulation: All embryos had aspirated to be included 5 μg cytochalasine B mL^{-1} of working medium. All micromanipulations were performed using a Nikon (Eclipse Ti) inverted microscope with phase contrast optics. Micromanipulators were attached to Ependoff motorized controls. In this study, two different types of pippete were used.

One of them was holding pippete which used to hold the embyo in proper position which had internal diameter 15 μM and angle 20° (OD:20 μM). The other one is aspiration pippete which used gentle extruction single blastomere from eight-cells embro which had an internal diameter 15 μM and angle 35° (OD:100 μM). All embryos had biopsied to be included 5 μg cytochalasine B mL^{-1} of working medium (Wilton and Trounson, 1989).

Experimental design: Experimental study had deviced two groups:

- Biopsied group
- Control group

After biopsy embryo culture: At post biopsy stage, all of biopsied embryos were immediately transferred to embryo culture medium. Each embryos of biopsied were cultured in 10 μL drops of SAGE medium supplemented with 5% CO_2, 5% O_2 and 90% air at 37°C for up to blastocyst stage (Bagis *et al*., 2009).

Fluorescent staining of total cell number: Throughout *in vitro* culture of post biopsied and non-biopsied blastocyst stage was observed under inverted microscope (Zeiss Axiovert 35 M). Total cell numbers of expanded blastocysts were evaluated using bisbenzimide (Hoechst 33342; B-2261) fluorescent DNA staining technique (Rall, 1987; Bagis *et al*., 2003).

Statistical analysis: The experiments were performed at least four replications. The data were analyzed using SPSS (Statistical Package Social Sciences, Version 10.0) for Windows (MS). Blastocyst viability rate and total blastocyst nuclei number was analyzed by Paired t-test. The p-value used to determine significance in all test was 0.05.

RESULTS AND DISCUSSION

There are many methods for removal of cells from pre-implantation embryos including zona drilling using acidified solutions (Handyside *et al*., 1990), zona thinning, aspiration using a fine micropipette (Wilton and Trounson, 1989) and blastomere displacement. In the present study, it has been demonstrated that mouse embryos can be biopsied with a simple aspiration procedure. Post-biopsy embryos and control embryos were cultured for 48 h in QABM medium (4 mg mL^{-1} BSA Fraction V) in 5% CO_2 and 95% air until expanded blastocyt stage. In this study, for the first time, biopsied mouse embryos were cultured in the QABM medium and embryo development rate was successful (Bagis *et al*., 2009). A single blastomere from each of 267 embryos was removed; only two out of 269 embryos were destroyed during manipulation. After biopsy and culture *in vitro*, 267 biopsied embryos out of 234 (88.32±7.5%) reached the expanded blastocysts developed from the biopsy group and total cell number mean 67.8±17.2% were recorded in the experiment group and 126 non biopsied embryos out of 118 (94.4±7.78%) expanded blastocysts developed

Table 1: Developmental potential of eight-cell mouse embryos after biopsy

Groups (8 cell embryos)	Before aspiration total 8 cell embryos	After aspiration total living 8 cell embryos (%)	No. of 8 cell embryos cultured	No. of expanded blastocysts (%)	Blastocyst total cell number (mean±SEM)
Aspiration	269	267 (99.25%)	267	234/267 (88.32±7.5)[a]	67.8±17.2
Control	126	126	126	118/126 (94.4±7.78)[a]	70.0±15.4

Groups with different superscripts in the same column are not significantly different: p>0.05. The percentages represent mean±SEM (p = 0.05)

from control embryos and total cell number mean 70±15.4% was recorded in the control group. In the present study, there was no difference in blastocysts developmental rates at post biopsy group and control group (p = 0.05) (Table 1). The removal of a blastomere was caused very little trauma to the embryos. It did not alter developmental potential *in vitro* after biopsy 88.3% (267 of 269) of the biopsied embryos reached the transferable stage. Furthermore, biopsy had no detrimental effect on pregnancy rates achieved using only biopsied embryos for transfer.

Some of the biopsied embryos, sex was determined in the 10 biopsied embryos. In 4 cases, very weak Y-specific signals were obtained which could not be identified unambiguously in 3 embryos amplification gave results that allowed sex to be determined. Some researchers advice to perform the biopsy method in Ca^{2+} inculuding medium (Handyside *et al*., 1990) while other researchers recommend the use of Ca^{2+}/Mg^{2+} free (Wilton and Trounson, 1989; Santalo *et al*., 1996; Illmensee *et al*., 2006; Krzyminska *et al*., 1990). The use of Ca^{2+}/Mg^{2+} free medium thus allows for an easier biopsy procedure during pre-implantation genetic diagnosis while it does not result in a loss of developmental potential of the embryo to the blastocyst stage.

Embryo biopsy suitable when embryos were pre-incubated for 60-90 min to decrease cell to cell contacts to 10 μL microdrops of Ca^{2+}/Mg^{2+} free bicarbonatte buffered QAM HTF (3 mg mL^{-1} BSA Fraction V) with HEPES. Use of Ca^{2+}/Mg^{2+} free medium has no loss effect on the viability of the biopsied embryos and their biopsied cells when used during relatively short time of exposure (Santalo *et al*., 1996).

Several studies clearly showed that biopsied on eight-cell mouse embryos were cultured successful to blastocyst development (Monk and Handyside, 1988; Wilton and Trounson, 1989; Krzyminska *et al*., 1990; Santalo *et al*., 1996; Illmensee *et al*., 2006). This study confirms that successful embryo biopsy can be carried out at eight-cell mouse embryo. There is no difference between experimental and control groups (Table 1). At the end of the embryo culture, total cell numbers per expanded blastocyst were determined using fluorescent DNA staining technique (Rall, 1987; Bagis *et al*., 2003). There is no difference between experimental nd control groups (Table 1).

CONCLUSION

In the present study, the method described here is a suitable method of blastomere aspiration on *in vivo* mouse embryos. The method have no affect on the survival and quality of pre-implantation mouse embryos after biopsy however, resulted *in vitro* development rates not difference from that of controls for blastomere biopsy method. The procedure may be applicable to other mammalian species.

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