EVALUATION OF DEVELOPMENTAL CHARACTERISTICS OF SINGLE BLASTOMERE BIOPSIED FROM 8-CELL MOUSE EMBRYO DURING PREIMPLANTATION DEVELOPMENT

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Abstract. In this research, we evaluated the preimplantation development, embryo morphology, the time of cell division and the cell number of the blastocyst stage of single mouse blastomeres biopsied from 8-cell embryos. One blastomere was biopsied from each 8-cell embryo using micromanipulation system with XYClone laser. After biopsy, the single blastomeres and biopsied embryos were cultured together in the in vitro development (IVD) medium until the blastocyst stage. Our data shows that, one blastomere could be developed to the blastocyst stage with approximately 6 cells ± 0.5 and the remaining embryo could be cultured normally to the blastocyst stage with 95 cells ± 4.9 (P < 0.05). However, the percentage of biopsied blastomere that have developed to the morula and blastocyst stage is lower than that of biopsied and non-biopsied embryos. The results also show that the time taken for biopsied blastomere to form morula and blastocyst was the same as that of biopsied embryos although the cell number was much lower. In conclusion, single blastomeres collected from the 8-cell stage mouse embryos can develop into morula and blastocyst with the same time. Thus, the time for embryo compaction and blastocyst formation was depended on the time of fertilization. These results provide potential benefits for preimplantation genetic diagnosis (PGD) in infertile couples at risk of genetic diseases as well as opening a novel approach for the establishment of stem cell line from biopsied blastomere combined with PGD in future research.

Keywords: Biopsy, blastomere, developmental characteristics, preimplantation development, 8-cell mouse embryos.

1. INTRODUCTION

It has been 32 years since the first successful application of preimplantation genetic diagnosis (PGD) to humans for the detection of males prone to X-linked adrenoleukodystrophy and X-linked mental retardation (Handyside et al., 1990). Until now, PGD has been employed to identify the status of the embryos produced by assisted reproductive technologies (ART). Specifically, it is prenatal diagnosis that can detect single gene disorders or chromosomal abnormalities in embryonic genetics that might subsequently put the embryos at risk of low implanted rate, resulting in the miscarriage of embryos or a child with a genetic disorder and/or physical disabilities (Sullivan-pyke et

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This process is typically performed on day 3 of embryo development in which a single blastomere is biopsied (biopsied blastomere) out of an 8-cell embryo (biopsied embryo) for genetic analysis. If the test result shows normal genetics, the embryo will be transferred to the mother’s uterus on day 5, initializing the implantation development (Stern, 2014).

Single blastomere derived from 8-cell embryos was used for PGD using different genetic technologies such as PCR, DNA microarray, or next-generation sequencing. In fact, the characteristics of such single blastomere have not been fully studied yet although many studies have been progressed at various stages of embryo growth. In 1959, Tarkowski reported that the blastomere derived from 2-cell embryos could become full-term (Tarkowski et al., 1959). In the splitting experiments performed on 4-cell stage human embryos, it was proven that four individual blastomeres can flexibly develop to blastocyst stage, giving rise to TE and ICM cells (Van de Velde et al., 2008). Therefore, it was concluded that totipotent characteristics of single blastomere remain from 2-cell to 4-cell embryos (Maemura et al., 2021). According to Kryminska et al. (1990), biopsies of 4-cell mouse embryos could lead to poor blastocyst formation and lower implantation rate, while biopsied 8-cell embryos developed normally. Interestingly, single blastomere derived from 2-cell to 8-cell mouse embryo could also develop as normal embryo into the blastocyst stage although there was also a demand for an optimal culture system to accelerate and enhance its developmental potency (Amri et al., 2016). This study also observes that at the 8-cell stage, if the separated single blastomeres have poor morphology, they can only generate trophoblastic vesicles instead of ICM. Similarly, based on the assessment of ICM cells and embryo growth, single blastomere biopsied from 8-cell embryos show some impairments in epiblast and primitive endoderm formation in vitro (Maemura et al., 2021).

Such restricted findings have raised a question about what would happen if these single blastomeres possessed valuable attributes that are significant to biomedical research. Therefore, in this research, the single blastomere will be separated from 8-cell stage embryos to examine its comprehensive characteristics including the morphology, developmental stage, the time form to morula and blastocyst, and cell numbers at the blastocyst stage. Furthermore, to avoid ethical issues, this research uses mice as an experimental unit and the blastomere biopsy is conducted at late 8-cell stage to ensure that the totipotency has disappeared.

If we can generate valid data and understand the developmental potency of biopsied single blastomeres through this research, there will be positive signs for ART and stem cell therapy in the future. In particular, the single blastomere separated from 8-cell mouse embryos can be continuously cultured until the blastocyst stage, then one single blastomere is extracted for PGD analysis. The other cells could be utilized for the establishment of embryonic stem cells (myESCs) without destroying the blastocysts, which brings hope to stem cell therapy for many cellular disorders that the child may suffer throughout their lives and overcomes ethical problems for the use of human embryos. As a result, we can achieve dual purposes when performing PGD in ART.
Therefore, if this research shows some signs of progress, we can achieve dual purposes as mentioned above when performing PGD in ART in human.

2. MATERIALS AND METHODS

2.1. Collection of 8-cell mouse embryos

Female mice were injected intraperitoneally with 5 IU of pregnant mare serum gonadotropin (PMSG) and 5 IU of human chorionic gonadotropin (hCG) 48 hours later to induce super-ovulation. Female ICR mice that had been treated with 2 kinds of hormones were immediately mated with male ICR mice during the night. A vaginal plug was observed in female mice next morning. The presence of the vaginal plug indicates successful mating of female and male mice approximately 15 hours later hCG injection.

After 40 hours, female mice with vaginal plugs were sacrificed by cervical separation for fertilized embryos. The oviducts were cut out and then transferred into HEPES-mCZB droplets in a Petri dish (60 x 10 mm). The location of the infundibulum was found at early position of the oviduct. The 2-cell embryos were obtained by flushing pipette through each infundibulum of oviduct. The collected embryos were then carefully washed in HEPES-mCZB medium and transferred in mCZB medium supplemented with 5 % BSA covered with mineral oil. The selected embryos were cultured at 37 °C, in 5 % CO₂, a humidified medium in a petri dish (35 x 10 mm) for further development. After 36-42 hours from in vitro culture of 2-cell embryos, the embryos at the 8-cell stage were utilized in the blastomere biopsy.

2.2. Isolation of single blastomere at the 8-cell embryos

Biopsy was performed under a micromanipulator system with XYClone laser (Hamilton Thorne) at 8-cell mouse embryos. After 36-42 hours of in vitro culture, the 2-cell stage embryos were developed to the blastocyst stage and are used for biopsy. First, 8-cell embryos were turned around by biopsy pipette to find out the best position and the suitable blastomere to withdraw from this embryo. Each 8-cell embryo was kept by a holding pipette. The distance between cell membrane and zona pellucida was large enough to perforate the zona membrane with XYClone laser and did not affect the quality of the embryo for the future development. Selected blastomeres were focused. The slit of zone pellucida was opened by using XYClone laser until a suitable hole for entering biopsied pipette. The optimal wavelength of XYClone laser was approximately 250 nm. From the hole already opened of the zona pellucida, a single blastomere per 8-cell embryo was aspirated slowly by using a biopsy pipette. The success of biopsy was assessed by the integrity and absence of cell membrane rupture of one biopsied blastomere. Following the biopsy, single blastomeres and biopsied embryos were obtained from 8-cell embryos.

After that, biopsied embryos and biopsied blastomeres were recovered for at least 20 minutes in the droplet of HEPES-mCZB medium at room temperature. Both were transferred into the same droplet containing mCZB medium supplemented with 5 % BSA covered with mineral oil and incubated at 37 °C, 5 % CO₂, in a humidified atmosphere. Finally, biopsied embryos and biopsied blastomeres were cultured and recorded next 2 or 3 days to reach the morula and blastocyst stage.
2.3. Preimplantation development of biopsied blastomeres and biopsied embryos

After the biopsy, both biopsied blastomeres and biopsied embryos were cultured in the same droplet of IVD culture medium supplemented with 5% BSA and stored at 37 °C, 5% CO₂ in a humidified medium. The embryo culture was carried out in droplets on a standard petri dish (35 x 10 mm) covered with mineral oil. These embryos were evaluated and recorded everyday under micromanipulator system for developmental efficiency in terms of morphology and time. The intact mouse embryos derived from the 2-cell fertilized embryos (non-biopsied embryos) were used as group of positive control for examining preimplantation development of biopsied blastomere and biopsied embryos.

2.4. Cell counts of blastocysts derived from biopsied blastomeres and biopsied embryos

The cell number was examined by DAPI (4,6-diamidino2-phenylindole) staining at blastocyst stage of isolated blastomeres and biopsied embryos. First, the blastocysts derived from biopsied blastomeres were fixed with 2% paraformaldehyde and blastocysts derived from biopsied embryos were fixed with 4% paraformaldehyde for 30 minutes at room temperature. After fixing, the blastocysts were rinsed twice in PBS-BSA 1% for 10 minutes. Second, permeabilization was performed using 0.1% Triton X-100 for 2 hours. After Triton treatment, the blastocysts then were washed twice in PBS-BSA 1% for 10 minutes. Next, the selected blastocysts were then stained the nuclei with 2 g/mL DAPI for 30 minutes at 4 °C and rinsed twice with PBS-BSA 1% for 10 minutes. Finally, the samples were put on a glass slide with glycerol and identified cell numbers in developed blastocyst under fluorescent microscopy. The cells were counted and recorded to evaluate the quality of blastocysts derived from biopsied blastomere and biopsied embryo.
2.5. Statistical analysis

The experiments were repeated at least six times for biological replications. The results were shown as means ± standard error of the mean. The percentage data for embryonic development and cell counts per blastocysts were carried out by One-way analysis of variance (ANOVA) with IBM SPSS Statistical Software version 20. For all analyses, P < 0.05 was considered to be statistically significant.

3. RESULTS AND DISCUSSION

3.1. Preimplantation development of biopsied blastomeres and biopsied embryos after biopsy

Table 1. Developmental competence of biopsied blastomeres and biopsied embryos to the blastocyst stage

<table>
<thead>
<tr>
<th></th>
<th>No. of 8-cell embryos examined</th>
<th>No. of embryos at day 1* (Morula)</th>
<th>No. of embryos at day 2* (Early blastocyst)</th>
<th>No. of embryos at day 3* (Expanded blastocyst)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biopsied blastomeres</td>
<td>65</td>
<td>50 (76.9) a</td>
<td>39 (60.0) a</td>
<td>32 (49.2) a</td>
</tr>
<tr>
<td>Biopsied embryos</td>
<td>65</td>
<td>62 (95.3) b</td>
<td>61 (93.0) b</td>
<td>60 (92.3) b</td>
</tr>
<tr>
<td>Non-biopsied** embryos</td>
<td>60</td>
<td>58 (96.7) b</td>
<td>58 (96.7) b</td>
<td>58 (96.7) b</td>
</tr>
</tbody>
</table>

* The day before biopsy. This experiment was repeated 6 times, a, b Values in columns with different superscripts differ significantly (P < 0.05). **Intact in vivo fertilized embryos were the positive control group

The development of blastomeres after being isolated from the 8-cell embryos was examined daily until day 1, 2, and 3 after biopsy and the results are shown in Table 1. The results showed that the percentage of biopsied embryos and non-biopsied embryos developed to the blastocyst stage (92.3 % and 96.7 %, respectively) was significantly higher than in the group of biopsied blastomeres (49.2 %, P < 0.05). However, as shown in Table 1, there was no significant difference in blastocyst formation between non-biopsied embryos and biopsied embryos (P > 0.05). The low rate of single blastomere developed to the blastocyst stage might be caused by biopsy technique as well as the collection of single blastomere is very difficult after biopsy separation because of its small size and difficult to control by mouth pipetting. This experiment also showed that the time that biopsied blastomeres and biopsied embryos form to morula and blastocyst was the same.

3.2. The timing of morula and blastocyst formation and morphology of biopsied blastomeres and biopsied embryos during preimplantation

The results from Table 1 and Figure 2 show that the developmental time and morphological features of biopsied blastomeres and biopsied embryos after biopsy at the 8-cell stage mouse embryos. Both were cultured in the same droplet of IVM medium for estimation of the embryo division time and the morphological changes after biopsy. There
was no difference between the time and morphology of biopsied embryos and biopsied blastocysts on preimplantation development. When the biopsied embryos developed into blastocysts, the biopsied blastomeres obtained from 8-cell embryos still underwent blastocyst forming. On day 2 and day 3 (from biopsied time), biopsied embryos reached the hatching blastocyst with a cavity and embryonic cells were hatching from the already hole which opened zona-pellucida after biopsy. The majority of biopsied blastomeres developed normally during preimplantation and shared a common feature of biopsied embryos with mostly large vesicles and expansion of blastocele. The blastomere blastocysts were following the biological clock of compaction on day 1 after biopsy and cavitation on day 2 and fully developed blastocysts on day 3. The size of the blastocysts derived from the biopsied blastomeres from day 2 to day 3 after biopsy was approximately four times smaller than the normal mouse blastocysts (Van de Velde et al., 2008). These results showed that although the size of biopsied embryos is smaller than that of biopsied embryos, the time of compaction, blastocele cavity formation is the same between single biopsied blastomere, biopsied embryo and non-biopsied embryos (Figure 2).

![Figure 2](image)

**Figure 2.** Developmental morphologies of biopsied blastomeres, biopsied embryos and non-biopsied embryos at day 1, day 2, and day 3 after biopsy. (A-A2) Blastocyst derived from biopsied embryo (***) and biopsied blastomere (*) at day 1, 2, and 3 after biopsy. (B-B2) Development of non-biopsied from morula to blastocyst. Scale bar = 50 µm.

### 3.3. Quality of blastocyst derived from single blastomere and blastomere-biopsied embryo

Along with developmental competence, the total cell number in blastocyst derived from biopsied blastomeres and biopsied embryos was also investigated by staining the nuclei with DAPI. As described in Figure 3, there was significant difference in number of cells between the biopsied embryos and biopsied blastomeres at the blastocyst stage. The rate of cell number at blastocyst was substantially higher in biopsied embryos group (95 cells ± 4.9) than in biopsied blastomeres group (6 cells ± 0.5), but the same as in non-biopsied embryos group (101 cells ± 1.8). As the results, from taking out isolated blastomere of 8-cell mouse embryos, single blastomeres can develop in vitro to the blastocyst stage with approximately six cells per blastocysts.
<table>
<thead>
<tr>
<th></th>
<th>No. of cell at blastocyst</th>
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</thead>
<tbody>
<tr>
<td>Biopsied blastomeres</td>
<td>(6 ± 0.5) a</td>
</tr>
<tr>
<td>Biopsied embryos</td>
<td>(95 ± 4.9) b</td>
</tr>
<tr>
<td>Non-biopsied embryos</td>
<td>(101 ± 1.8) b</td>
</tr>
</tbody>
</table>

**Figure 3.** The cell number in the blastocyst stage on day 3 after biopsy of biopsied blastomere, biopsied embryo and non-biopsied embryo groups. (A-A1) non-biopsied embryo, (B-B1) biopsied embryo, (C-C1) biopsied blastomere. Blue is DAPI staining. Scale bar = 30µm.  

**4. CONCLUSION**

The results of this study showed that biopsied blastomere obtained from 8-cell embryo could develop to blastocyst, but the rate was lower than that of biopsied embryos. Biopsied embryos developed well to blastocysts with a high cell count comparable to non-biopsied embryos. The results also showed that the formation of morula and blastocyst was not depended on the number of cells but depended on the time of fertilization. These results give us the opportunity to use single biopsied blastomere not only for PGD but also to develop stem cell lines for the future stem cell therapeutic applications.

**REFERENCES**


ĐÁNH GIÁ ĐẶC DIỂM PHÁT TRIỂN CỦA TẾ BÀO PHÔI ĐƠN SINH THIỆT TỪ PHÔI CHƯỢT 8 TẾ BÀO TRONG GIAI ĐOẠN PHÁT TRIỂN TIỀN LÀM TỔ

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Tóm tắt. Trong nghiên cứu này, chúng tôi đã đánh giá sự phát triển tiền làm tổ, hình thái phổ, thời gian phân chia tế bào và số lượng tế bào của tế bào phổ được sinh thiết từ phổ 8 tế bào và phổ sau khi đã được sinh thiết. Tế bào phổ đơn được sinh thiết từ phổ 8 tế bào bằng hệ thống vi phẩu thuật kết hợp với laser Xyclone. Sau khi sinh thiết, tế bào phổ đơn và phổ sinh thiết được nuôi cấy cùng trong một làn môi trong suốt để phát triển đến phổ nang. Kết quả của nghiên cứu cho thấy rằng, tế bào phổ đơn được sinh thiết từ phổ 8 tế bào có thể phát triển đến phổ đầu và phổ nang, tuy nhiên tỷ lệ hấp thụ sơ vô với phổ não cho tế bào sau khi sinh thiết. Mặc dù tế bào phổ đơn có thể phát triển đến phổ nang, nhưng số lượng tế bào rất thấp (6 tế bào ± 0,5) so với phổ não cho tế bào (95 tế bào ± 4,9). Kết quả cũng cho thấy thời gian để tế bào phổ đơn hình thành phổ đầu và phổ nang cùng giống như thời gian của phổ qua tế bào mặc dù số lượng tế bào thấp hơn nhiều (P < 0,05). Kết luận, các phổ nang dạng đơn được thu thập từ phổ chuẩn ở giai đoạn 8 tế bào có thể phát triển thành phổ đầu và phổ nang. Phổ 7 tế bào sau khi đã sinh thiết phát triển đến phổ nang với tỷ lệ cao và chất lượng tốt tương đương với phổ không sinh thiết. Kết quả này mang lại những lợi ích tiềm năng cho việc chuẩn đoán di truyền trước khi cấy ghép (PGD) ở các cặp vợ chồng hiếm muộn có nguy cơ mắc bệnh di truyền cùng như mở ra một phương pháp mới để thử lặp dòng tế bào gốc từ một tế bào phổ đơn sau khi sinh thiết kết hợp với kỹ thuật PGD khi nghiên cứu trong tương lai.

Từ khóa: Đặc điểm phát triển, phát triển tiền làm tổ, phổ chuẩn 8 tế bào, sinh thiết, tế bào phổ đơn.

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