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# Comparison of the development of mouse embryos manipulated with different biopsy techniques

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**Abstract**: Preimplantation genetic diagnosis is the detection of inherited diseases and the sex of embryos before implantation in the practice of human medicine as well as in veterinary medicine. The introduction of experimental animal embryo biopsy techniques has been a milestone in the developmental process of preimplantation genetic diagnosis techniques. The aim of the present study was to evaluate in vivo and in vitro development of embryos after biopsy in an experimental mouse model and to perform comparisons across different biopsy techniques (blastomere biopsy and trophectoderm biopsy). At the end of the study, no significant difference was observed between the blastomere biopsy group and the control group in terms of in vitro development, embryo quality, and fetal development, whereas embryo quality and in vivo development were negatively affected in the trophectoderm biopsy group ($P < 0.05$).

**Key words:** Mouse, embryo, biopsy, blastomere, microblade, trophectoderm

## 1. Introduction

The aim of prenatal (preimplantation) genetic diagnosis (PGD) is to detect some inherited diseases and the sex of the embryo before implantation of the embryo to the recipient. The detection of genetic diseases and chromosomal abnormalities and sex analysis in embryos are the main areas of use in mice, humans, and farm animals. Polymerase chain reaction (PCR) and fluorescence in situ hybridization (FISH) are diagnostic techniques used to evaluate biopsy materials (1).

In PGD, first and second polar body biopsy and blastomere isolation are performed in embryos at different stages of development, and trophectoderm biopsy is performed in embryos at the blastocyst stage. The biopsy of mammalian embryos was performed for the first time in 1959 (2). Gardner and Edwards (3) developed a biopsy procedure to identify sex in rabbit embryos during blastocyst stage. Moreover, Willadsen (4) described the use of the blastomere aspiration technique in sheep and performed successful blastomere biopsies in in vitro mouse embryos at different stages of development (5,6). The microblade biopsy technique has been used in bovine and rhesus monkey embryos at the blastocyst stage (7,8). On the other hand, twin embryos were produced by bisecting the embryo with a microblade using mouse as a model (9,10). Cenariu et al. used needle, aspiration, and microblade biopsy techniques in bovine embryos (11). Sex identification can be performed in farm animals after embryo biopsy following fertilization. This procedure will be of strategic importance in the production planning of farms. Bredbacka et al. performed blastomere aspiration biopsy and blastocyst microblade biopsy techniques in bovine embryos (12). The advantages and disadvantages of these biopsy methods should be considered when planning PGD studies. There are no data in the literature on the use of trophectoderm biopsy using microblade technique in mouse blastocysts.

The aim of the present study was to evaluate in vitro development ratios, quality, and in vivo development ratio of embryos after biopsy in an experimental mouse model and to perform comparisons across different biopsy techniques (aspiration biopsy and trophectoderm biopsy by microblade).

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## 2. Materials and methods

The Uludağ University Animal Experiments Local Ethics Committee approved the study and confirmed that the experiments were conducted in accordance with ethical principles.

### 2.1. Superovulation and embryo harvest

CB6F1 (C57BL⁄6 × BALB⁄c) female mice were injected intraperitoneally with 10 IU of pregnant mare serum gonadotropin (Sigma PMSG). Superovulation protocol was completed by intraperitoneal administration of 7.5 IU of human chorionic gonadotropin (Organon hCG) 48 h after the injection, and female mice were mated with male CB6F1 (C57BL⁄6 × BALB⁄C) mice (13). The superovulated females were sacrificed 68–72 h after hCG administration. The oviducts of the sacrificed mice were washed with HEPES-buffered 3 mg/mL BSA (Sigma A3311) supplemented with Quinn's Human Tubal Fluid (HTF), and eight-cell embryos were harvested (5,14).

### 2.2. Micromanipulation

All micromanipulations were performed at 40× magnification using a Nikon Eclipse Te inverted microscope coupled with Eppendorf Transfer Man/Nk 2 micromanipulators. HEPES-buffered Quinn's HTF containing 3 mg/mL BSA + 5 µg/mL cytochalasin B ($Ca^{2+}$/$Mg^{2+}$-free) was used as the biopsy medium for blastomere biopsy (5,14) and HEPES-buffered Quinn's HTF containing 3 mg/mL BSA ($Ca^{2+}$/$Mg^{2+}$-free) was used as biopsy medium for the trophectoderm biopsy (9,10).

#### 2.2.1. Blastomere aspiration biopsy

Compact 8-cell mouse embryos cultured in incubators at 37 °C containing 5% $CO_2$ and 5% $O_2$ were placed under a stereomicroscope in 10 µL of HEPES-buffered Quinn's HTF ($Ca^{2+}$/$Mg^{2+}$-free) supplemented with 3 mg/mL BSA + 5 µg/mL cytochalasin B. An Eppendorf Cell Tram Air embryo-handling pipette console and an embryo-handling pipette with a 35° angle and an interior diameter of 15 µm and exterior diameter of 100 µm were placed in the left micromanipulator. An Eppendorf Cell Tram Vario embryo manipulation pipette console and an Eppendorf embryo biopsy pipette with a 20° angle and an interior diameter of 15 µm and exterior diameter of 20 µm were placed in the right micromanipulator. The embryos were monitored for 30 min, and biopsy was performed after interblastomere connections were loosened and decompacted. After immobilizing the embryos at a 9 o'clock position under an inverted microscope at 40× magnification, the zona pellucida was gently punctured with a biopsy pipette introduced from the 3 o'clock direction. After passing the perivitelline space, a single blastomere was aspirated from the decompacted eight-cell embryo with an oil-controlled biopsy micromanipulator (5,14).

#### 2.2.2. Trophectoderm biopsy

The eight-cell embryos harvested as described above were cultured for 24 h in incubators at 37 °C containing 5% $CO_2$, 5% $O_2$, and 4 mg/mL BSA under conditions of high humidity until the blastocyst stage in Quinn's Advantage Blastocyst Medium (SAGE-QBM). Microfeather K-715 30° and 15° microblades connected to the Eppendorf automated consoles were used in the biopsy cutting procedure. The blastocysts were washed in biopsy medium three times and subsequently transferred to 50 µL of biopsy medium in a 150 mm × 15 mm petri dish that was prepared for embryo cutting, in such a way that a single embryo was placed in each drop. The embryos were rinsed in QAM with HEPES ($Ca^{2+}$/$Mg^{2+}$-free), and they became more adhesive to the surface of the petri. To limit the movement of the embryos during the manipulation, parallel lines were drawn on the petri dish's surface with the aid of the microblade. The cutting procedure was then performed on the embryos placed between the parallel lines, using an inverted microscope at 40× and a microblade. The cutting procedure was performed on the trophectoderm cells that were encircling the blastocyst that had been placed at the opposite pole of the inner cell mass. Following the biopsy procedure the microblade was washed and cleaned using distilled water, 70% ethanol, and biopsy medium (9–11,15).

### 2.3. In vitro culture after biopsy

All embryos were transferred to embryo culture medium in order to evaluate the in vitro embryo development following the biopsy. SAGE-QBM supplemented with 4 mg/mL BSA was used for in vitro embryo culture medium. The embryos were cultured in an incubator with high humidity at 37 °C containing 5% $CO_2$ and 5% $O_2$ for 48 h and 24 h after blastomere biopsy and trophectoderm biopsy until the expanded blastocyst stage, respectively. The rates of in vitro development at the end of the process were evaluated (13).

### 2.4. Detection of total cell count with fluorescein staining

In order to evaluate the quality of the expanded blastocysts that developed following the manipulations, total cells were determined by staining with the fluorescein DNA staining technique (Hoechst 33342; B-2261) under a fluorescein microscope. The embryos at the blastocyst stage in the in vitro culture medium were selected and transferred to the prepared slides using a mouth-controlled pipette; they were then stored for 10 min in the dark after they were covered with aluminum foil. Total cell counts were determined under a Zeiss/Axiovert fluorescence microscope (13,16).

### 2.5. Embryo transfer

CD1 female recipient mice mated with vasectomized CB6F1 males that were observed to have a vaginal plaque were accepted as being at 0–0.5 days and they were used for uterine embryo transfer at 2.5 or 3.5 days following

this time period. During the 6–14 developed expanded blastocyst stage embryos were transferred into one uterine horn (right) of recipient mice and kept until 13–15 days of pregnancy for the in vivo development rate (absorption site and living fetuses) (13). Three animals were used in each group for embryo transfer.

**2.6. Statistical analysis of the results**

SPSS 17.0 for Microsoft Windows was used to perform statistical analysis of the results. ANOVA and independent t-tests were used to evaluate the differences between the groups. Each experiment was replicated at least three times.

## 3. Results

A total of 152 eight-cell embryos in the blastomere aspiration biopsy group and 63 eight-cell embryos in the control group were transferred to in vitro culture medium and cultured in an incubator at 37 °C containing 5% $CO_2$ and 5% $O_2$ under conditions of high humidity for 48 h. The number of blastocyst development following in vitro culture was 121 (81.02 ± 13.61%) and 62 (96.37 ± 3.17%) in the experiment and control groups, respectively (Table 1). In vitro culture rates were similar in the blastomere biopsy and control groups ($P > 0.05$). The mean total cell count was 50 ± 6.00 in the blastomere aspiration biopsy group and 50 ± 10.39 in the control group (Table 1), and there was no statistically significant difference between the two groups ($P > 0.05$). Seventy-nine blastocysts in the trophectoderm microblade biopsy group and 28 blastocysts in the control group were transferred to in vitro culture medium and incubated for 24 h in an incubator at 37 °C containing 5% $CO_2$ and 5% $O_2$. Following in vitro culture, the number of blastocysts was observed to be 69 (86.96 ± 2.39%) and 23 (93.33 ± 11.54%), respectively, in the experiment and control groups (Table 1). The rates of blastocyst development in the trophectoderm biopsy and control groups were similar ($P > 0.05$). The total cell count was 26.66 ± 5.770 in the trophectoderm biopsy group and 55.33 ± 11.015 in the control group (Table 1), and the difference between the groups was statistically significant ($P < 0.05$).

Thirty-six blastocysts developing in the blastomere biopsy group and 30 blastocysts developing in the control group were transferred into the uteruses of 3 recipient CD1 mice that were detected to have vaginal plaques on days 2.5–3.5. The recipient mice were sacrificed between days 13 and 15 in order to evaluate the rate of in vivo development. Subsequently, implantation areas were evaluated. Nine implantation areas (25.00%) were detected as a result of uterine embryo transfer in the blastomere biopsy group, while only 7 showed fetal development (19.44%). In the control group, on the other hand, 8 (26.66%) implantation areas were observed, while only 6 showed fetal development (20.00%) (Table 2). No significant difference was found in the number of implantation areas as a result of the embryo transfer that was performed to evaluate in vivo development between this group and the control group ($P > 0.05$). Similar results were found when fetal development rates were compared to the control group ($P > 0.05$).

Thirty-two blastocysts in the trophectoderm biopsy group and 22 blastocysts in the control group were selected to be transferred into the uteruses of 3 recipient CD1 mice that were found to have vaginal plaques at days 2.5–3.5. The implantation areas were evaluated in the recipient mice sacrificed between days 13 and 15 in order to analyze the rate of in vivo development. In the trophectoderm group, seven implantation areas (21.88%) were detected following embryo transfer and no fetal development (0.00%) was observed in those areas. In the control group, on the other hand, 13 areas of implantation were observed (59.09%), but fetal development was present in only four (18.18%) (Table 2). As a result of embryo transfer, no significant difference was found in the number of implantation areas compared to the control group ($P > 0.05$). However, fetal development rates were significantly different in the biopsy and control groups and no fetal development was observed to occur in the biopsy group ($P < 0.05$).

**Table 1.** Evaluation of in vitro development in biopsy and control groups.

| Embryo stage | Group | Number of embryos (n) | Number of in vitro developed embryos (%) | Mean number of cells |
|---|---|---|---|---|
| Eight-cell | Blastomere biopsy | 152 | 121[a] (81.02 ± 13.61%) | 50 ± 6.00[a] |
| | Control | 63 | 62[a] (96.37 ± 3.17%) | 50 ± 10.39[a] |
| Blastocyst | Trophectoderm biopsy | 79 | 69[a] (86.96 ± 2.39%) | 26.66 ± 5.770[b] |
| | Control | 28 | 23[a] (93.33 ± 11.54%) | 55.33 ± 11.015[a] |

[ab]: Differences between values marked with different letters in the same column are significant ($P < 0.05$).

**Table 2.** Evaluation of in vivo development in biopsy and control groups.

| Embryo stage | Group | Number of blastocysts transferred | Pregnant/recipient numbers | Number of implantation areas (%) | Number of fetal development areas (%) |
|---|---|---|---|---|---|
| Eight-cell | Blastomere biopsy | 36 | 2/3 | 9 (25.00 ± 36.46)[a] | 7 (19.44 ± 26.92)[a] |
| | Control | 30 | 2/3 | 8 (26.66 ± 23.09)[a] | 6 (20.00 ± 20)[a] |
| Blastocyst | Trophectoderm biopsy | 32 | 3/3 | 7 (21.88 ± 31.17)[a] | 0 (0.00 ± 0)[b] |
| | Control | 22 | 3/3 | 13 (59.09 ± 27.74)[a] | 4 (18.18 ± 6.36)[a] |

[ab]: Differences between values marked with different characters in the same column are significant ($P < 0.05$).

## 4. Discussion

In this study, in vitro development rates in the blastomere biopsy and control (no biopsy) groups in mouse embryos at the eight-cell stage and in the trophectoderm biopsy and control (no biopsy) groups in the blastocyst stage were found to be 81.02%, 96.37%, 86.96%, and 93.33%, respectively, and the mean total cell count in the developed embryos was found to be 50, 50, 26.66, and 55.33, respectively. The rate of implantation following transfer in the same order was found to be 25%, 26.66%, 21.88%, and 59.09%, respectively, and the rate of fetal development was found to be 19.44%, 20.00%, 0.00%, and 18.18%, respectively. The biopsy techniques used in this present study were found to have affected in vitro development rates. However, total cell counts and implantation rates were similar for the blastomere aspiration technique and the control group. On the other hand, the trophectoderm biopsy technique was found to result in lower numbers in terms of total cell number and in vivo fetal development rates compared to the control group.

Some investigators introduced the use of biopsy medium ($Ca^{2+}$- and $Mg^{2+}$-free) to loosen intercellular bonds and facilitate manipulations (5,6,10,11). The biopsy medium ($Ca^{2+}$- and $Mg^{2+}$-free) that was used in the present study impaired the integrity of the tight junctions, which are the main elements of intercellular bonds, and thus intercellular bonds were loosened and blastomeres were obtained in as short a time as 2–3 min. Results obtained from the present study revealed that the use of $Ca^{2+}$- and $Mg^{2+}$-free medium in the biopsy procedure did not affect in vitro development negatively.

Wilton and Trounson (17) observed that obtaining a single blastomere from four-cell mouse embryos with aspiration biopsy technique had no negative effect on in vitro development, and they reported that they found an in vitro development rate of 94% and 98% in the biopsy and control groups, respectively, following 48 h of culture. In the same study, in vivo development rate of embryos biopsied after transfer to recipient was found to be lower compared to the control group (53.1% and 81.8%, respectively).

Bodo et al. (18), in their biopsy study performed with eight-cell mouse embryos, demonstrated no statistical difference between the rates of blastocyst development in the biopsy and control groups. When the number of cells in the biopsy group and the control group were compared, late development was observed in the biopsy group since the number in that group was lower. Although the rates of implantation were similar, development of the embryos biopsied in the uterus on day 9 of pregnancy was observed to have been delayed for 12–24 h. No statistically significant differences were found in the in vitro blastocyst development or total number of cells in in vivo development between the blastomere biopsy group and the control group ($P > 0.05$).

Krzyminska et al. (14) evaluated in vitro and in vivo development following biopsies in four-cell, eight-cell, and morula stages of mouse embryos. HTF medium with HEPES buffer ($Ca^{2+}$- and $Mg^{2+}$-free) supplemented with 5–7.5 μg/mL cytochalasin B + 3 mg/mL BSA was used as the biopsy medium. Blastomeres were kept in the biopsy medium for 20–30 min to loosen the bonds between the blastomeres and prevent compacting. As a result of that study, the application when performed in the eight-cell stage was reported to result in the least damage. When the biopsy group at the eight-cell stage was compared to the control group, in vitro development and implantation rates were found to be comparable, at 95% and 99%, and 71% and 71%, respectively. Similarly, the embryos were placed in biopsy medium for 30 min to loosen the bonds between the blastomeres to prevent compacting. In the study presented here, the rates of embryo development with the aspiration technique used in the eight-cell stage and the development rates in the study by Krzyminska et al. (13) were found to be similar.

Park et al. (19) reported that blastomere biopsies performed in eight-cell bovine embryos had no negative effect on blastocyst development. No significant differences were found in the blastocyst development rates between

the blastomere aspiration group and the control groups; thus, the investigators reported that this technique could be used in bovine embryos. In the present study, blastomere biopsy had no negative effect on blastocyst development in eight-cell mouse embryos. When the development rates following in vitro culture in the blastomere biopsy group and control group were compared, no significant difference was found between the groups ($P > 0.05$). Similar results were also found when the total number of cells of the developed blastocysts was compared with the controls ($P > 0.05$). Likewise, the results of the embryo transfer performed to evaluate the in vivo development showed no significant differences in the implantation rates and fetal development rates between the blastomere biopsy group and the control group ($P > 0.05$).

The trophectoderm biopsies were performed using different techniques. Summers et al. (20) evaluated in vivo embryo development following a trophectoderm biopsy in marmoset monkey embryos. Using micromanipulation techniques, the researchers performed a laceration in the zona pellucida, in the opposite direction to the inner cell mass. Herniation from the performed lacerations was seen in 20% of the embryos 24 h after the manipulation and in 50% after 48 h. Herniated trophectoderm cells were excised at the neck level of herniation and the embryos were transferred to the recipients, but this technique was not used in the present study. In the present study, trophectoderm cells were directly excised without the development of herniation in the opposite direction to the inner cell mass.

Wang et al. (9) investigated the capacity of the development of the embryos, which they had divided into two equal parts with a microblade, in mouse morula and blastocysts. The in vitro development rate was found to be higher in the group in which biopsy was performed at the blastocyst stage compared to the group with biopsy performed at the morula stage. The number of cells obtained from the blastocysts developed from the divided embryos was found to be the half the number of cells in the whole embryos used as the control group. In that report, the implantation rate was a little lower in the divided embryos compared to the control group, while a serious decline was observed in fetal development. Similarly, in the present study, fetal development was not observed in the trophectoderm biopsy group.

Carson et al. (21) obtained embryos at the morula stage from B6DF1 mice and used trophectoderm microbiopsy methods (microblade/incision, trophectoderm aspiration, slit/excision, and hatch/excision). In their results, in vitro development rates of biopsied blastocysts of the slit/excision and hatch/excision techniques were higher than those of the microblade/incision and trophectoderm aspiration techniques. In vitro development rates after microblade trophectoderm biopsy were notably lower compared to other techniques and there were no live-born mice. In our study, we obtained embryos at the eight-cell stage from CB6F1 mice and used a similar microblade. However, in our results, the rates of in vitro culture in the trophectoderm biopsy and control groups were found to be similar ($P > 0.05$). In our study, we also observed that total cells were determined by performing staining. Similar to the work of Carson et al., we did not observe any live-born developments in the trophectoderm biopsy group.

Bagis et al. (13) obtained embryos from CB6F1 mice and cultured them in SAGE medium until the blastocyst stage. Their result for mean total cell number was 58.9. We used the same mouse strain and embryo culture protocol. Similarly, in the present study, the mean total cell count in the developed embryos was found to be 50, 50, 26.66, and 55.33, respectively. The lowest total cell number was obtained with the trophectoderm biopsy technique due to cell loss.

Bredbacka et al. (12) used a microblade biopsy protocol on bovine blastocysts produced with IVF for trophectoderm biopsy. They used a technique similar to that used in the present study in bovine embryos. In order to gain experience and have a model for human and bovine embryos, it has been shown here that the technique may be used in mouse embryos as an experimental model. Dokras et al. (22) reported that human blastocysts were more resistant to lacerations when human and mouse embryos were compared in terms of the ease of applying micromanipulations and viability rates. Following trophectoderm biopsy in 1050 human blastocysts, McArthur et al. (23) found that 974 blastocysts (93%) continued their viability. Nevertheless, in this present study, no statistically significant difference was found in the in vitro culture rates following trophectoderm biopsy performed on the embryos in the blastocyst stage when compared with the control group ($P > 0.05$). However, when the mean total number of cells of the developed blastocysts was compared with the control group, the difference was found to be significant ($P < 0.05$). As a result of the embryo transfer performed to evaluate in vivo development, no significant difference was found in the implantation areas in the trophectoderm group compared to the controls ($P > 0.05$). However, no fetal development occurred in the biopsy group and the difference between the fetal development rates was found to be significant ($P < 0.05$). It was considered that the reason for no fetal development was due to a high rate of cell loss in the trophectoderm biopsy group.

For healthy fetal development, there should be only minimal damage during the manipulations of the embryos. In the present study, blastomere aspiration technique appears as a superior technique compared to blastomere

and trophectoderm biopsy techniques due to the ease of application and reliability of results. In conclusion, because of its ease of use, this technique is considered useful in biopsy studies of other farm animals, as well as human embryos, especially with its comparable results in in vitro/in vivo development rates compared to controls, and in particular for the conditions of in vitro embryo production.

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