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Blastocyst development of 4-cell mouse embryos after laser destruction of one blastomere with or without its microsurgical removal

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Abstract

Aim: To study the rate of blastocyst formation in 4-cell mouse embryos after laser destruction of one blastomere, with or without microsurgical removal of the destroyed blastomere.

Methods: Mouse embryos were randomly allocated to two control and two experimented groups. Control embryos were either non-manipulated (117 embryos) or underwent laser ablation of zona only (114 embryos). Experimented embryos had laser destruction of zona and the adjacent blastomeres. Destroyed blastomeres were either left *in situ* (115 embryos) or were microsurgically removed (107 embryos). They were cultured in sequential media for 72 h and were assessed for cleavage/morula arrest and blastocyst formation rates.

Results: Embryos arrested at cleavage/morula stages were higher when destroyed blastomeres remained *in situ* (30.4%) than when they were immediately removed (15.0%, $P < 0.05$). Blastocysts in the group with immediate removal of the destroyed blastomeres (85%) were significantly higher than when destroyed blastomeres were left *in situ* (69.6%, $P < 0.05$). Blastocyst formation in the repaired embryos was significantly lower than the non-manipulated (91.5%) and the manipulated controls (94.8%, $P < 0.05$). Hatching blastocysts were highest in control embryos with zonal ablation (72.8%). Proportions of hatching/hatched blastocysts in embryos, with or without removal of destroyed blastomeres, were not significantly different (39.3% and 33.9%, respectively). The percentage of embryonic loss during an attempt at microsurgical repair was 6.1%.

Conclusion: Microsurgical removal of destroyed blastomere was effective in restoring blastocyst development. It could reduce the rate of cleavage/morula arrest.

Key words: blastocyst formation, destroyed blastomere, hatching, laser ablation, microsurgical removal.

Introduction

Fragmentation is frequently observed in human embryos obtained in the laboratory. The causes of fragmentation are still unknown, but the most prevalent theory is programmed cell death or apoptosis.[1] As such, some types of fragmentation are believed to arise from complete disintegration of one or more blastomeres.[1,2] Degenerate blastomeres and fragmentation are also frequently observed in animal and human embryos before the pre-implantation period and after freezing and thawing.[3]

It is still unknown whether degenerate blastomeres and fragmentation are associated with a decreased

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developmental and survival rate of the embryos. However, several reports have shown that these embryos may have a reduction in the rate of blastocyst formation, a smaller inner cell mass, a decrease in hatching rate and a reduction in implantation potential.[4–8]

Alikami *et al*.[4] evaluated the effects of blastomere degeneration in mouse embryos. A micromanipulation technique was used first to destroy one or two blastomeres of 4-cell mouse embryos and later to remove the degenerate cells. The results showed that three-quarter embryos had a lower hatching rate than intact 4-cell embryos (53% and 91%, respectively). When half-quarter embryos were compared to those that had degenerate blastomeres removed, the hatching rates were 3% and 55% in protein-free medium, respectively. After repair of half embryos by microsurgical technique, a more dramatic increase in hatching was achieved. Furthermore, immediate removal of destroyed blastomeres resulted in a higher hatching rate than those half-embryos that were cultured overnight before repair.[4]

In this study, the effect of degenerate blastomeres on embryonic development up to the blastocyst stage was explored using a mouse model. Intact 4-cell embryos, with or without a hole in the zona, served as controls. In the study groups, one of the four blastomeres was destroyed by a laser beam. The damaged blastomeres were left *in situ* or were microsurgically removed to see if it could restore normal embryonic development.

Materials and Methods

Five- to seven-week old ICR female mice were superovulated by i.p. injection of 5 IU pregnant mare serum gonadotropin (Sigma Chemical, St Louis, MO, USA), followed 48 h later by i.p. injection of 5 IU human chorionic gonadotropin (N.V. Organon, Holland). Immediately after the second injection, females were paired with ICR males. They were checked for mating by the presence of vaginal plugs 16 h later.

Approximately 24 h later, the oviducts of mated female mice were removed. Two-cell embryos were flushed from the oviducts with phosphate buffered solution (Gibco BRL, Gaithersburg, MD, USA) containing human serum albumin (Sigma). They were cultured in small drops of cleavage medium (Cook, Australia) under oil (Medicult, Denmark) at 37°C in an atmosphere of 6% CO_2, 5% O_2 and 89% N_2 for 8–12 h. Only 4-cell embryos with equal and regular blastomeres without any fragmentation were selected for the experiment. Embryos were randomly divided into two controls (C) and two experimented groups (E) as:

- C 1: Non-manipulated embryos (117 embryos)
- C 2: Embryos with a laser-induced zonal hole (114 embryos)
- E 1: Embryos with laser destruction of zona and one adjacent cell (115 embryos)
- E 2: Same as E1 with immediate microsurgical removal of the destroyed cells (114 embryos)

Micromanipulation and laser microsurgery

In this experiment, the laser was generated by a non-contact solid-state diode source (Saturn Laser System, Research Instruments, UK). The machine emitted an infrared laser beam with a wavelength of 1480 nm at a power of 140 mW.

Four-cell embryos were placed individually in a 5-μL drop of cleavage medium under oil. No more than three embryos were manipulated in the same dish at a time to avoid a drastic change in pH and temperature. The dish was placed on the heated microscopic stage. In the control group (C2), an area of zona far away from any blastomere was targeted to ensure that the laser beam would not come into contact with the blastomeres. A pulse length of 3.5 ms was selected to create a small hole in the zona (Fig. 1). In the study groups (E1 and E2), an area of zona in contact with one blastomere was selected. The laser beam was aimed at the point where the blastomere touched the inner surface of the zona. The same pulse length of 3.5 ms was used to simultaneously destroy the blastomere and to create a hole in the zona (Fig. 2). In our pilot study, we found

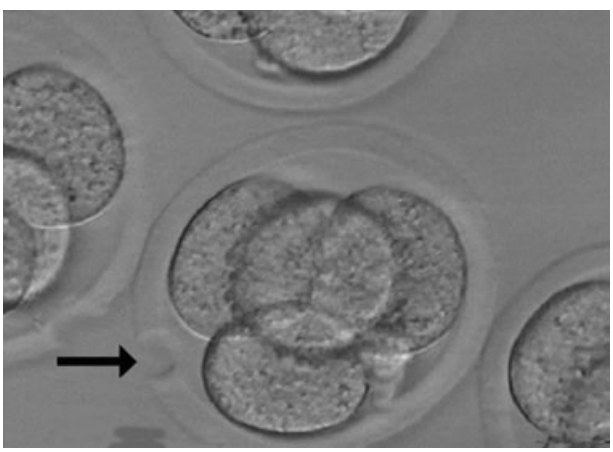

Figure 1 Zonal ablation in control (C2) (arrow).

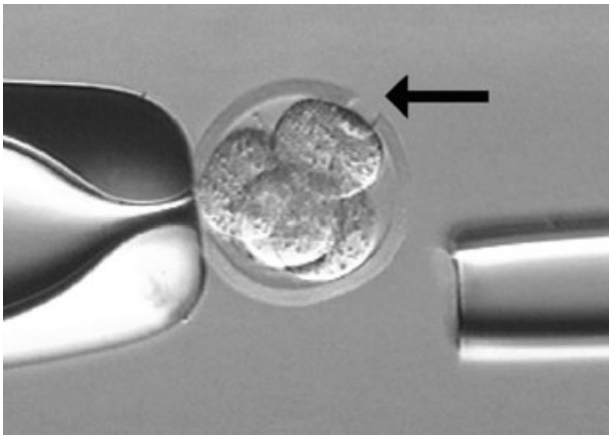

Figure 2 Laser destruction of zona and a blastomere (arrow).

that this method was always fatal to the lasered blastomeres.

The destroyed blastomeres were left *in situ* in one group (E1) or were removed within a few minutes after the destruction by a laser beam in the other group (E2). Aspiration was carefully performed using a SAS-SE air microsyringe (Research Instrument) and a biopsy pipette with an internal diameter of 35 µm (Fig. 3).

Mouse embryos culture

Control and micromanipulated embryos were washed twice in cleavage medium. They were then cultured in groups of 10 in 25-µL drops of cleavage medium under oil at 37°C in a humidified atmosphere of 6% CO_2, 5% O_2 and 89% N_2. After 24 h of culture, embryos with further cleavage were transferred into 25-µL drops of

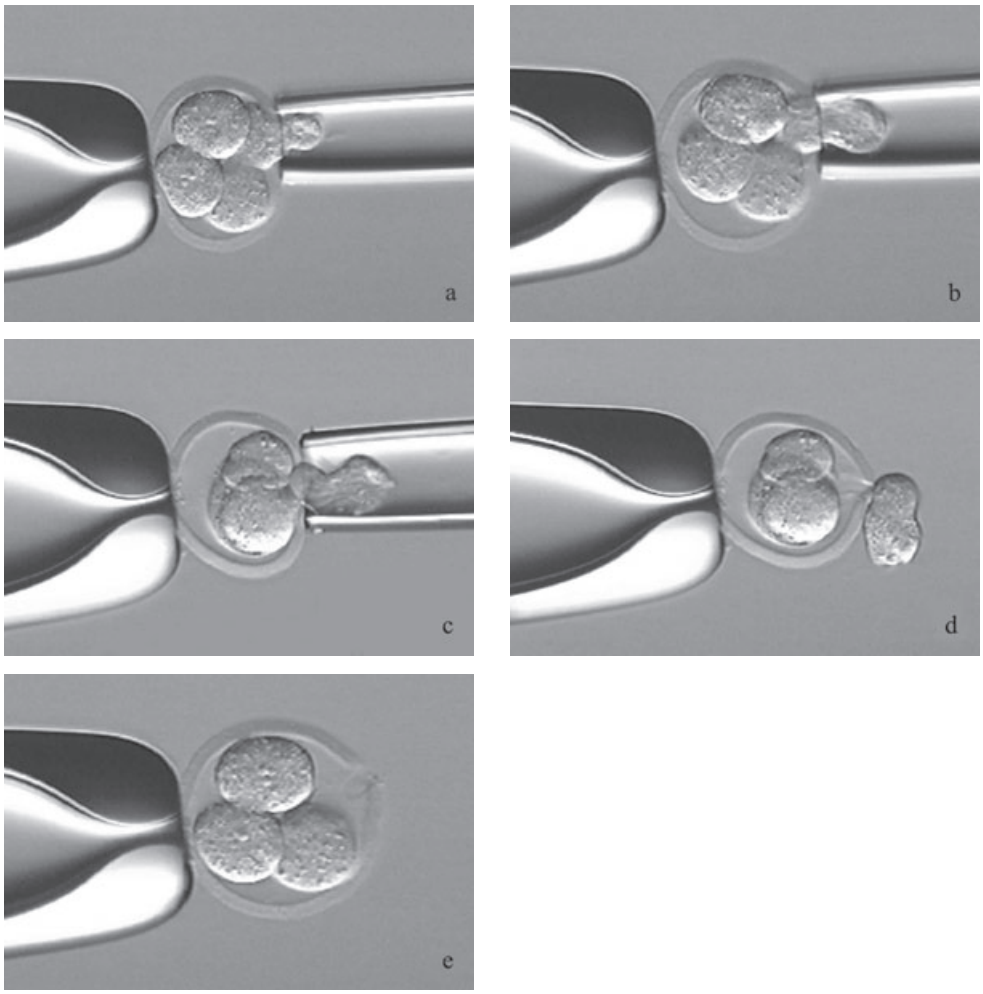

Figure 3 Microsurgical removal of destroyed blastomere from 4-cell mouse embryo (a–d) and end result (e).

Table 1 Further development of 4-cell embryos in controls and experimented groups

Group	C1 *n* = 117	C2 *n* = 114	E1 *n* = 115	E2 *n* = 107
Cleavage arrest	0	0	0	0
Morula arrest	10 (8.5%)	6 (5.2%)	35 (30.4%)	16 (15.0%)
Blastocysts	107 (91.5%)	108 (94.8%)	80 (69.6%)	91 (85.0%)

Table 2 Quality of blastocysts in control and experimented groups

Groups	C1 *n* = 117	C2 *n* = 114	E1 *n* = 115	E2 *n* = 107
Early/full blastocyst	15 (12.8%)	7 (6.1%)	20 (17.4%)	22 (20.6%)
Expanding blastocyst	32 (27.4%)	18 (15.8%)	21 (18.3%)	27 (25.2%)
Hatching/hatched blastocyst	60 (51.3%)	83 (72.8%)	39 (33.9%)	42 (39.3%)

blastocyst medium (Cook) for another 48 h. The numbers of embryos arrested at the cleavage/morula stages were recorded. The numbers and grading of blastocysts were assessed after 48 h in culture.

Blastocysts were scored based on their degrees of expansion and hatching as suggested by Gardner *et al*.[9] as follow:

- Stage 1: Early blastocyst – blastocoel less than half of the volume of the embryo
- Stage 2: Partial blastocyst – blastocoel equal to or greater than half of the volume of the embryo
- Stage 3: Full blastocyst – blastocoel completely filled the embryo
- Stage 4: Expanded blastocyst – blastocoel volume larger than that of the early embryo with a thinning zona
- Stage 5: Hatching blastocyst – trophectoderm starting to herniate through the zona
- Stage 6: Hatched blastocyst – blastocyst completely escaped from the zona

Statistical analysis

The proportions of blastocyst formation after 72 h of culture in all groups were compared by a χ^2 test using the Stata program (Stata, College Station, TX, USA). The proportions of early/full blastocyst, expanding blastocyst and hatching/hatched blastocyst were also compared using a χ^2 test. A *P*-value of <0.05 was considered statistically significant.

Results

Four-hundred and sixty embryos with four equal and regular blastomeres without any cytoplasmic fragmentation and with intact zona pellucida were included in the study. All manipulated embryos survived the laser ablation procedures. During the microsurgical attempt to aspirate the single destroyed blastomere, two or more blastomeres were accidentally removed from seven embryos (6.1%) in the E2 group. These embryos either died immediately or had cleavage arrest the next day. As a result, 107 embryos remained in the E2 group. The time taken to perform zonal ablation followed by blastomere removal was, on average, 20 s.

After 24 h of culture, all embryos underwent further cleavage and were transferred into blastocyst medium. After another 48 h, development into blastocysts was found to be 91.5% in the non-manipulated embryos (C1) and 94.8% in the zonal ablated embryos, respectively (C2). In the study groups, removal of the destroyed blastomere (E2) resulted in a significantly higher rate of blastocyst formation when compared to the group (E1) without removal of destroyed blastomeres (85% vs 69.6%, $\chi^2 = 7.5075$, $P = 0.006$). Rates of blastocyst development in the two study groups were significantly lower than those of controls ($\chi^2 = 34.1647$, d.f. = 3, $P < 0.0001$; Table 1).

When blastocyst quality was taken into consideration (Table 2), 4×3 χ^2 test showed that there was a significant difference among the four groups ($\chi^2 = 10.5240$, $P = 0.005$). Partition of χ^2 revealed that the C2 group (control embryos with zonal ablation) had significantly more hatching and hatched blastocysts than the other three groups. Blastocyst quality in the three remaining groups was not significantly different.

Discussion

Alikami *et al*.[4] destroyed one or two blastomeres in 4-cell mouse embryos by tearing the cytoplasmic

membranes and the zona pellucida with partial zona dissection (PZD) needles. Using this PZD technique, it must have been difficult, if not impossible, to control the extent of damage inflicted upon the embryos and zona in their study. To overcome this problem, we employed a non-contact laser with a power of 140 mW at a constant pulse of 3.5 ms to destroy a blastomere and the adjacent zona in a single hit. Theoretically, the extent of damage to embryos and zona in our study should be more uniform.

In this study, the control group (C2) with laser ablation of the zona pellucida had the highest blastocyst formation rate (94.8%). This was compatible with blastocyst rates reported in other studies (84.7–98.0%).[10,11] However, statistical comparison revealed no significant difference in the rate of blastocyst formation among the non-manipulated (C1) and the laser-ablated controls (C2). Our study therefore confirmed previous reports[10–12] that laser ablation of the zona pellucida had no significant effect on subsequent blastocyst development. When the rates of hatching blastocyst were compared, that in the laser-ablated control (C2) was significantly higher than those in the other three groups. This result agreed with the report by Park *et al*.[13] who showed an increase in the hatching rate of bovine blastocysts after laser zona drilling. Neev *et al*.[14] also observed that the hatching process occurred earlier in lasered embryos compared to non-manipulated controls.

Both our study and that by Alikami *et al*.[4] confirmed that blastocyst formation rates dramatically increased after repair of the embryos by microsurgical removal of dead blastomeres. However, the rate of morula arrested in our study group, in which degenerate blastomeres were left under the zona, was higher than in their study (30.4% vs 14%). The hatching blastocyst rate was also lower (33.9% vs 82%). This could be due to many factors. For example, mouse embryos were collected at the 2-cell stage in our study and were cultured *in vitro* until they became four cells, while Alikami *et al*.[4] employed *in vivo* developed 4-cell embryos for their experiment. In addition, we had to transport mouse embryos in a transport incubator over a distance from the place of collection to the human *in vitro* fertilization (IVF) unit, in order to use the laser and micromanipulator facilities. In this study we cultured mouse embryos in a 5% O_2 atmosphere as this had been shown in a previous study to result in improved embryo development without regard to type of strains.[15] However, the culture medium and culture condition were optimized for human but not necessarily for mouse embryos.

It is still a matter of speculation as to how the removal of fragments/degenerate blastomeres from certain embryos helps their survival and improves their subsequent development. Fragments and degenerated blastomeres may undergo secondary necrosis, and release toxic metabolites.[1] This can create an unfavorable environment to other nearby blastomeres and cause them to deteriorate and eventually arrest.[4,7,8] Alternatively, fragments/degenerate blastomeres may be situated between normal blastomeres and interfere with cell-to-cell contact. In turn, they can hinder compaction and ultimately inhibit blastocyst development.[1] On the other hand, embryos with a severe degree of fragmentation/degeneration will not be rescued by fragment removal because the amount of cytoplasm available for blastomere division is markedly reduced.[2]

Microsurgical removal of fragments/degenerate blastomeres was a safe procedure when performed by an experienced embryologist.[2,3] In our study, seven out of 114 four-cell embryos (6.1%) accidentally had one or more blastomeres removed along with the dead blastomeres. These embryos either died or subsequently arrested. We could not comment on our rate of embryo damage during the microsurgical repair, as previous studies did not report their numbers. It suffices to mention that the risk of embryonic loss should be taken into account before any attempt at fragment removal be considered. In this study, we did efficacy analysis by excluding damaged embryos from the study group. If we were to include them as arrested embryos, the rate of blastocyst formation would be 80/115 and 91/114 in the E1 and E2 groups, respectively. Effectiveness analysis would then show a trend towards an improvement in blastocyst formation ($\chi^2 = 3.1861$, $P = 0.074$).

In our study, accidental aspiration of normal blastomeres was usually encountered when embryos began to show compaction. Compaction also made it difficult to thoroughly remove all cellular debris. On some occasions, we had to be satisfied with the removal of approximately 90% of the destroyed blastomere in order to avoid inadvertent injury to other blastomeres. We anticipated that the risk of damage to embryos would be less if aspiration were done in calcium and magnesium free biopsy medium. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered medium should also be considered if more than a few embryos were to be micromanipulated at a time. In the study group (E1) in which

destroyed blastomeres were left under the zona, we occasionally observed spontaneous loss of degenerate blastomeres through the laser-ablated holes during subsequent development. However, the numbers were too few to conclude whether this spontaneous loss affected subsequent blastocyst development.

Another limitation in our study was that we only measured the outcome by counting the proportion of blastocyst developments. It is possible that fragments/degenerate cells may lead to a reduction in cell numbers in the resulting blastocysts. In fact, cell numbers in the inner cell mass and trophectoderm are important indicators of a blastocyst's health.[16] To shed light on this important parameter, total cell count in the blastocysts should be performed. It would be even better to check blastocyst viability by transferring them into pseudopregnant mice, and counting the number of implantation sites/fetus in each uterine horn.

Although our study was performed on fresh embryos, we believe that removal of degenerate blastomeres from frozen–thawed embryos should also be beneficial. Technically, the micromanipulation procedure will be similar to that for fresh embryos.[3] In fact, a recent randomized trial did show a significant increase in pregnancy and implantation rates when necrotic blastomeres were removed from partially damaged frozen–thawed human embryos before transfer.[3]

Acknowledgments

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