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The effect of the site of laser zona opening on the complete hatching of mouse blastocysts and their cell numbers

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Objective: We studied the effect of the site of laser zona opening on the complete hatching of mouse blastocysts and the cell numbers of the completely hatched blastocysts.
Methods: Mouse blastocysts were randomly allocated to the inner cell mass (ICM) group (zona opening performed at the site of the ICM, n = 125), the trophectoderm (TE) group (zona opening performed opposite to the ICM, n = 125) and the control group (no zona opening, n = 125).
Results: The rate of complete hatching of the blastocysts was not significantly different in the ICM and the TE group (84.8% vs 80.8%, respectively; $p = 0.402$), but was significantly lower in the control group (51.2%, $p < 0.001$). The cell numbers in the completely hatched blastocysts were comparable in the control group, the ICM group, and the TE group (69 ± 19.3, 74 ± 15.7, and 71 ± 16.8, respectively; $p = 0.680$).
Conclusion: These findings indicate that the site of laser zona opening did not influence the rate of complete hatching of mouse blastocysts or their cell numbers.

Keywords: Blastocyst; Hatching; Inner cell mass; Lasers; Zona pellucida

Introduction

The success of *in vitro* fertilization depends on many factors, one of which is the ability of the embryo to hatch from the zona pellucida (ZP) [1]. Hatching involves cell proliferation and increased internal hydrostatic pressure, which results in blastocyst expansion and thinning of the ZP [1,2]. This process occurs in conjunction with the secretion of lysin protease, from the trophectoderm (TE) cells and/or uterus, to degrade the ZP [3]. Artificial opening of the zona, known as assisted hatching (AH), has been advocated as a way of increasing *in vitro* fertilization implantation and pregnancy rates. Although many studies have been carried out to evaluate this proposal, no consensus exists on the benefits and risks of AH [3-6]. Debate continues about whether partial zona thinning is preferable to full-thickness AH, as it has been claimed to promote hatching without the potential drawbacks of the full-thickness approach. In our previous study, we found that zonal thinning by 90% of zonal thickness for a distance of one-quarter of the zonal circumference enhanced the hatching rate but increased the rate of incompletely hatched blastocysts [7]. This was confirmed in a similar study by Tinney et al. [8]. In a randomized trial of laser thinning for a distance of one-quarter of the zonal circumference, Valojerdi et al. [9] reported a significant decrease in clinical pregnancy and implantation rates in vitrified/warmed human embryos, in comparison with non-hatched controls.

The effect of the AH site on the hatching process has received considerable attention. Observational studies [10,11] have suggested

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that the natural hatching site in humans is near the inner cell mass (ICM), whereas the natural hatching site in mice is opposite to the ICM. Furthermore, some studies have reported that AH performed in the ZP opposite to the ICM caused more trapping of human blastocysts than AH performed near the ICM [2,12]. However, the numbers of observed blastocysts in these studies were too few to enable a definitive conclusion regarding whether the appropriate rupture site in the ZP was necessary for the hatching process to be successful [2,13].

In practice, embryologists usually perform laser-AH in an area away from the ICM to avoid any possible detrimental effect on the embryo. Similarly, in preimplantation genetic diagnosis, the ZP is usually opened opposite to the ICM for TE biopsy. If the site of zona opening does affect the hatching process, artificial zona openings performed without regard to the natural hatching site will, in theory, negatively affect the hatching process. In this study, we used a mouse model to study the effect of the site of zona opening on the rate of complete hatching of mouse blastocysts and the cell counts of the completely hatched blastocysts.

Methods

Outbred Institute of Cancer Research (ICR) mice were purchased from the National Animal Institute, Mahidol University, Nakornpathom, Thailand. They were kept at the Animal Husbandry Unit, Faculty of Medicine, Chiang Mai University, in a well-ventilated room at 25°C ± 2°C with 60% to 70% humidity and controlled cycles of 12 hours of light and 12 hours of dark. Before the experiment, the mice were left undisturbed for 5 days to eliminate the effects of stress caused by transportation. The Animal Ethics Committee of the Faculty of Medicine, Chiang Mai University approved the use of mice for this study (protocol no. 37/2556). We followed international guidelines for ethical conduct in the care and use of animals for research [14].

1. Collection of two-cell embryos

Two-cell embryos were collected as previously described [7]. In brief, 5- to 7-week-old ICR female mice were superovulated with 10 IU of pregnant mare serum gonadotropin (Sigma, St. Louis, MO, USA) intraperitoneally, followed 48 hours later by 10 IU of human chorionic gonadotropin (hCG) (Pregnyl, Organon, Oss, The Netherlands). After the hCG treatment, the female mice were paired with 12- to 20-week old ICR males. They were checked for mating by the presence of a vaginal plug 16 hours later, and sacrificed by cervical vertebra dislocation 36 hours after the hCG treatment. Two-cell embryos were flushed from the oviducts with phosphate-buffered saline (Gibco, New York, NY, USA) containing 0.5% bovine serum albumin (Sigma), and washed twice in cleavage medium (Cook, Brisbane, Australia) under paraffin oil (Medicult, Jyllinge, Denmark). They were cultured in groups of ten in 10-μL drops of cleavage medium until they became early blastocysts.

2. Interventions

In this study, early mouse blastocysts were divided into one control and two experimental groups: the ICM group, in which the zona was opened near the ICM, and the TE group, in which the zona was opened opposite to the ICM.

The ZP was opened using a XYClone laser system (Hamilton Thorne Biosciences, Beverly, MA, USA), attached to an inverted microscope (Eclipse TE300, Nikon, Tokyo, Japan) equipped with a heated stage (Kitazato, Fujinomiya, Japan). The machine emitted an infrared laser beam with a wavelength of 1,480 nm, at a power of 140 mW. The pulse was set to create a 20 μm hole in the mouse ZP.

Up to five early blastocysts were placed in a 10-μL drop of medium under oil and put on the microscopic stage at a time. The laser beam was fired once at the ZP to create a full-thickness opening of 20 μm. In the control group, blastocysts were placed on the stage for the same duration as the experimental groups, but the laser pulse was not performed.

3. Culture conditions and assessment of embryo development

After the intervention, groups of 10 embryos were cultured together in 10-μL drops of blastocyst medium (Cook) under paraffin oil, in an atmosphere of 6% CO_2, 5% O_2, and 89% N2 at 37°C. The embryos were cultured in this medium for up to 72 hours without medium renewal. Embryo development was observed 24, 48, and 72 hours after the intervention. The blastocysts were classified as early, partial, full, expanding, hatching, and hatched blastocysts, using the criteria of Gardner et al. [15] for human blastocyst development.

4. Differential staining of ICM and TE cells

Differential staining was performed on all completely hatched blastocysts, using the protocol described by Pampfer et al. [16]. In brief, the blastocysts were washed 3 to 4 times in calcium- and magnesium-free buffer before exposure to rabbit anti-mouse antibody (M5774, concentration 1:50, Sigma) for 30 minutes at 37°C. After washing, they were transferred into guinea pig complement serum (S1639, Sigma) with propidium iodide (P4170, Sigma) and bisbenzimide (B2261, Sigma) at 37°C for 10 to 15 minutes. The blastocysts were washed and transferred onto glass slides to allow them to air-dry. The slides were mounted in glycerol, and the cells in the ICM and the TE were counted under a Nikon E600 epifluorescence microscope equipped with the LUCIA FISH program (Laboratory Imaging, Prague, Czech Republic). The ICM nuclei were stained blue, while the TE nuclei showed an intense pink color. Mitotic nuclei were counted, but

dead and pyknotic nuclei were not counted.

5. Statistical analysis

STATA ver. 8.2 (Stata Co., College Station, TX, USA) was used to perform the statistical analysis. The chi-square test was used to compare the proportions of hatching blastocysts as well as the number of completely hatched blastocysts in the experimental and control groups at 24, 48, and 72 hours after the intervention. The Fisher exact test was used when any of the expected cell frequencies was below 5. The mean numbers of the ICM and the TE cells of the hatched blastocysts from different groups were compared by one-way analysis of variance (ANOVA), with the Scheffe post-hoc test as appropriate. Two-tailed *p*-values <0.05 were considered to indicate statistically significant differences.

Results

A total of 375 early blastocysts were included in the study. In both experimental groups, all blastocysts (125 of 125 and 125 of 125) had developed into hatching blastocysts by 48 hours after the intervention, and they initiated hatching through the artificial opening site. In contrast, only 121 out of 125 embryos in the controls developed into hatching blastocysts, which was a significantly lower rate than was observed in the experimental groups ($p=0.018$). The proportion of completely hatched blastocysts 72 hours after the intervention in the ICM group was not significantly different than the proportion observed in the TE group (106 of 125 and 101 of 125, respectively, $p=0.402$), but was significantly higher than was observed in the control group (64 of 125, $p<0.001$) (Table 1).

No significant differences were observed in the number of cells in the hatched blastocysts in the ICM (ANOVA test, $p=0.456$), the TE (ANOVA test, $p=0.411$), and the total cells (ANOVA test, $p=0.680$) in both the ICM and TE groups, or in the control groups (Table 2). Likewise, the ICM-to-TE ratio in the hatched blastocysts was not significantly different among the three groups (ANOVA test, $p=0.339$).

Discussion

Few studies have evaluated the effect of the AH site on the proportion of completely hatched blastocysts, and the available results remain inconclusive. A study by Miyata et al. [2] of vitrified-warmed human blastocysts showed that zona openings close to the ICM (embryonic pole) resulted in a higher rate of completely hatched blastocysts (68.8%, 11 of 16) than those performed opposite to the ICM (anembryonic pole; 12.5%, 2 of 11). The authors proposed that hatching did not occur randomly, but that the hatching process exhibited a degree of polarity. If this is true, it will have an impact on current practices of ZP opening. Clinically, AH is usually performed in cleavage-stage embryos when the location of the future ICM is not known. In cases of blastocyst biopsy, the zona is intentionally opened opposite to the ICM to obtain TE cells for genetic studies. Interestingly, a subsequent study by Ren et al. [13] showed a different result. In that study, a total of 160 vitrified-warmed blastocyst transfer cycles were randomly allocated to two AH groups. In 80 cycles, AH was performed near the ICM, and in another 80 cycles it was performed opposite to the ICM. The authors found no difference in the implanta-

Table 1. The rate of hatching and completely hatched blastocysts in the control group and the experimental groups

Outcome	Control group (n=125)	ICM group (n=125)	TE group (n=125)
Hatching rate[a]	121 (96.8)	125 (100)	125 (100)
Hatched rate[b]	64 (51.2)	106 (84.8)	101 (80.8)

Values are presented as number (%).
ICM, inner cell mass; TE, trophectoderm.
[a]Control vs. experimental groups (ICM or TE group), chi-square test, $p=0.018$; the hatching rate was observed 48 hours after the intervention; [b]Control vs. experimental groups (ICM or TE group), chi-square test, $p<0.001$; and for the ICM group vs. the TE group, chi-square test, $p=0.402$; the final hatched rate was observed 72 hours after the intervention.

Table 2. Numbers of cells in the ICM, TE, the ICM-to-TE ratio, total cells (ICM and TE) in the control, ICM, and TE groups

Parameter	Control group (n=44)	ICM group (n=89)	TE group (n=76)	*p*-value
ICM	21.82±10.60	25.20±11.05	23.74±8.98	0.456
TE	48.50±13.81	51.34±10.91	49.52±12.26	0.411
ICM-to-TE ratio	0.45±0.08	0.49±0.09	0.48±0.07	0.339
Total cell	69.06±19.34	74.01±15.76	71.57±16.80	0.680

Values are presented as mean ± standard deviation.
ICM, inner cell mass; TE, trophectoderm.
Some blastocysts were excluded due to accidental loss or poor staining.

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tion rate (51.1% vs. 53.6%) or the clinical pregnancy rate (63.8% vs. 67.5%) between the groups. Their study suggested that the site of AH had no effect on the pregnancy rate. The discrepancy between these studies may be due to many factors. The difference in the size of the zona opening, which was 20 μm in the study of Miyata et al. [2] and 50 μm in the study of Ren et al. [13], may have played a role. It is possible that a larger opening reduced the likelihood of trapping the blastocyst. Furthermore, the hatching process *in vivo* and *in vitro* may not be the same [13], as *in vivo* hatching is accompanied by intrauterine ZP-lytic activity that is absent in the *in vitro* environment [17,18].

This study focused on the site of laser zona opening and its effect on completely hatched blastocysts. A zona opening of 20 μm in size was employed, as our previous study showed that this size was optimal for promoting mouse blastocyst hatching [7]. Our study supports the view that the site of zona opening—either near or opposite to the ICM—has no influence on the complete hatching of mouse blastocysts. We also demonstrated that artificial zona opening was an effective method for enhancing the complete hatching of blastocysts.

In this study, the mean number of cells in the ICM and TE, as well as the ICM-to-TE ratio, were comparable in the hatched blastocysts in the experimental and control groups. These results do not agree with those previously reported by Montag et al. [19]. In their study, the blastocyst cell count significantly decreased after laser AH in comparison with the controls. However, they performed AH in two-cell embryos, while our study opened the zona in the early blastocyst stage. In our previous study [7], we also found a significant decrease in blastocyst cell count when laser AH was performed in the morula stage compared to controls. In light of these previous findings, we postulated that zona opening might have a detrimental effect on the number of cells in the blastocyst when it is performed too early during the cleavage stage. This unfavorable effect was not seen when the zona was opened after the embryos reached the early blastocyst stage. It is possible that premature contact of cleavage-stage embryos with the external environment could be harmful to their subsequent development. Alternatively, a higher number of cells, together with early fluid accumulation inside the early blastocysts, could make them less vulnerable than cleavage-stage embryos to the subtle thermal injury induced by the laser zona drilling. Further studies should be carried out to confirm our findings and to elucidate the mechanisms involved.

In conclusion, in this *in vitro* study, we found that the site of the laser zona opening had no effect on the complete hatching of mouse blastocysts or on the cell number of completely hatched blastocysts. Further studies are needed to explore *in vivo* effects on the transfer of embryos to the uterus in order to determine the implantation and pregnancy rates, because the mechanism of *in vivo* and *in vitro* hatching may be different. This study also has limitations in terms of whether the results can be extended to humans, as differences may exist among species in zonal thickness (6.5 μm in mice [20] versus 16 μm in humans [21]), composition, and other factors.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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