An improved mechanical technique for assisted hatching

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BACKGROUND: Varied clinical outcomes of assisted hatching (AH) have been reported. We attempt to investigate whether the size of the zona opening created by AH is adequate for blastocyst hatching, and, if not, set up a new method to improve it. METHODS: A new AH technique, long zona dissection (LZD), was established, and experiments were performed to compare the effects of different sizes of zona opening on complete hatching of blastocysts in mouse and human embryos in vitro. RESULTS: The LZD technique can create a long zona slit on early embryos, even blastocysts, with the slit size beyond two-thirds of zona diameter. Compared with three-dimensional partial zona dissection, LZD can significantly enhance the hatching speed and the rate of complete hatching of mouse blastocysts (93.9%). All (100%) human blastocysts completely hatched following LZD; however, when the slit size after AH was about two-fifths of zona diameter, more of the larger inner cell masses (ICM) became trapped by the zona opening during hatching than the smaller ICM (53.3 versus 12.5%, P < 0.01).

CONCLUSIONS: Zona opening of moderate size following AH is inadequate for the completion of blastocyst hatching in vitro; in some cases, however, it can be significantly improved by LZD.

Key words: assisted hatching/blastocyst/inner cell mass/safety/zona pellucida

Introduction

The successful hatching of the embryos is thought to be a key event in the implantation process. In IVF, thickening of zona pellucida (ZP) may arise correlating with basal FSH level and pre-ovulatory estradiol (Loret De Mola et al., 1997). Further, zona hardening was also thought to occur during in vitro culture (De Felici and Siracusa, 1982) or after cryopreservation (Carroll et al., 1990). Blastocyst hatching and implantation may be impaired in some patients whose ZP is too thick or hard (Cohen et al., 1992). The relatively low implantation rates of embryos resulting from IVF have been attributed at least in part to impaired hatching (Cohen, 1991). It has been hypothesized that assisted hatching (AH) may enhance embryo implantation, not only by mechanically facilitating the hatching process, but also by permitting early embryo endometrium contact. In clinic, AH has been verified to be effective on selected women with advanced age (Meldrum et al., 1998) and/or repeated IVF failure (Stein et al., 1995).

However, some other studies indicated no benefit to aged women by zona drilling AH (Bider et al., 1997) and laser AH (Horng et al., 2002). An increasing number of controversial clinical outcomes of AH have been reported and there is now debate as to whether AH really has advantages in defined subgroups of patients.

It has been shown that small holes resulting from application of mechanical zona dissection inhibited completion of the hatching process rather than improving implantation rates (Cohen and Feldberg, 1991). However, we further noted in vitro that a zona opening with usual/moderate size, in some cases, can also trap hatching blastocysts, making some blastocysts unable to be integrally hatched out. If this hatching trap can also take place in vivo, that would lead to the delay or the failure of complete hatching of blastocysts, and cause instability or failure of embryo implantation. We hypothesize that inappropriate size of zona opening would be a potential defect of conventional AH methods.

We now investigate a new AH technique to create a zona slit of controlled size, and perform experiments to test whether or not the zona opening of a moderate size, corresponding with the size of current AH methods, would be large enough for the complete hatching of blastocysts in vitro, and whether the new technique could improve the in vitro effect of hatching, as well as decrease the technical manipulation.
Materials and methods

Source of materials and embryo culture
Mouse embryos were derived from the lineage of KMB (Kun Ming Bai) mouse from Xiayang School of Medicine in Central South University. The animal care abided by the Guiding Principles of the Care and Use of Animals in China. Human embryos were collected from the couples who agreed to donate their spare embryos for research and had signed informed consent, which was approved by IVF Ethic Committee of Xiayang Hospital, after ICSI or IVF at the Reproductive Medical Center of Xiayang Hospital Central South University. Embryos were cultured in medium of micro-drops in tissue culture dish (Falcon 3001; Becton Dickinson, USA), overlaid with light mineral oil (Irvine Scientific, USA) and cultured in a 37°C humidified incubator (Forma Scientific, USA). Mouse embryos were cultured in series medium of InVitroCare (USA) at 5% CO₂ in air. Human embryos were cultured in series medium of Vitrolife (Sweden) at 6% CO₂ in air.

Manipulation using new technique
A new technique called ‘controlled zona dissection’ (CZD) was established as described in Figure 1. In the CZD, micro-pipettes are modified. The classical vertical opening of the holding pipette is altered to bevelled opening with an angle of ~65° (Figure 1) made by using a pipette puller (Narishige, Japan) and microforge (Japan). The tip of the hatching needle is blunted by a gentle heating on the microforge, and is thin at the front, just like a conventional ICSI needle that can also be used as the hatching needle in this new procedure. In the CZD, the procedure is modified as described in Figure 1. In brief, using the modified holding pipette with a bevelled opening can reduce the time required to force the needle through the embryo’s side, enabling easy and stable manipulation while puncturing the ZP from the side of the embryo, even using a blunted hatching needle. The blunted hatching needle can then push the embryonic cells aside with no damage to cells and so can create a large perivitelline space, even in the case of a blastocyst which usually has no perivitelline space. The hatching needle can puncture the zona to a controlled size, followed by dissecting the punctured zona with the arc of the needle cutting against the bottom of the dish. The ZP dissection by CZD needs only one attempt to obtain a slit of the ‘-’ type. We shall refer to CZD creating a slit size of about two-fifths of the embryo’s diameter as moderate zona dissection (MZD), and a slit size beyond two-thirds of the embryo’s diameter as long zona dissection (LZD).

The comparison of AH technique between LZD and 3D PZD on mouse embryo development and hatching
Mouse embryos were harvested on day 2 post-mating. Good quality embryos at 8-cell stage on day 3 were allocated to three groups at random: LZD-AH group, 3D PZD-AH group and control group with no AH. 3D PZD was referenced to Cieslak (1999) to make two cross-slits as ‘+’ (Cieslak et al., 1999) with each slit size of 20–30 µm on zona of mouse embryos. LZD on zona of mouse embryos created a slit size beyond 70 µm. Embryos following micromanipulation were observed for their rates of development to blastocysts, observable progressing hatching rates, and rates of complete hatching of blastocysts by days 4, 5 and 6 post-mating. Observable progressing hatching refers to blastocysts hatching in an observable progressing state with observable thinned zona and increased diameter under ×200 microscope magnification or cells hatching out of the zona.

The CZD-AH in human blastocysts
Fifty-nine human blastocysts were manipulated by AH at middle-blastocyst stage with a selective size of zona opening by CZD: LZD group with slit size beyond 100 µm and MZD group with slit size between 55 and 65 µm as described in Table II. Embryos were allocated to different groups at random with respect to quality but not with respect to number. Considering the very limited number of donated human embryos, more embryos were allocated to the MZD group, as we were more concerned about the disadvantage of zona opening with usual/moderate size. Embryo hatching was observed and photographs were taken from day 5 post-fertilization.

Figure 1. Procedure of controlled zona dissection. (A) The embryo is held at about the 8 o’clock position by modified holding pipette and the hatching needle with blunted tip pierces the zona pellucida (ZP) at about the 5 o’clock or half-past-5 position. A much larger perivitelline space can be obtained by pushing the cell membrane aside by using the tip of the needle as it is moved forward. (B) When the needle tip reaches the opposite ZP, it is withdrawn a little from the ZP, then the embryo is released from holding pipette. (C) The needle is used to position the embryo away from holding pipette, and the embryo is rotated. (D) A new site of the embryo is held, and the needle is pulled back into the perivitelline space (PVS) and is moved as in step A to pierce more ZP. Repeat from A to D and we can obtain a controlled size of pierced ZP span, or for embryos with large PVS, one step as in A can result in a controlled size of pierced ZP span. (E and F) The needle is then inserted into the opening of the holding pipette (E) deeply until the embryo is pushed to the curve of the needle (F). (G) The curve of the needle is pressed against the bottom of the dish to cut the pierced ZP. (H) For assisted hatching of a blastocyst, the zona opening site can be selected at the site opposite to the inner cell mass (ICM), near the ICM or other designated site.
Statistics
Statistical analysis was performed by two-tailed \( \chi^2 \)-test and Fisher’s exact test using the SSPS system. \( P < 0.05 \) was accepted as statistically significant.

Results
Zona span with controlled size can usually be pierced (Figure 2A and B) and cut as a slit within 1 min by CZD. In LZD, a slit may be achieved as long as half of the perimeter of the zona (Figure 2C) but with the width of slit opening being within \( \sim 5 \) \( \mu \)m. No blastomere was damaged by CZD in manipulation. Some blastocysts collapsed during CZD manipulation, but these blastocysts recovered and generally expanded within 4 h.

The rates of blastocyst formation among the three mouse embryo groups were not significantly different (not significant); however, rates of observable progressing hatching blastocysts were significantly enhanced in the LZD group, compared with the 3D PZD and control groups (100 versus 95.1 versus 90.7\%, \( P \leq 0.05 \), Table I). The rates of complete hatching of blastocysts on day 5 and day 6 among the three mouse embryo groups were significantly different. Complete hatching of blastocysts on day 4 was found only in the LZD group, while the earliest complete hatching of blastocysts in the 3D PZD group was found on day 5, and in the control group on day 6. Although the rate of complete hatching of blastocysts on day 6 in the 3D PZD group (62.0\%) was significantly higher than that of the control group (28.8\%), 33.1\% of hatching blastocysts in the 3D PZD group still failed to completely hatch on day 6. However, the LZD group achieved a rate of 93.9\% for complete hatching of blastocysts on day 6, which was significantly higher than that of the other two groups.

The long slit with size beyond 100 \( \mu \)m created by LZD can be largely opened to a large zona opening during blastocyst hatching (Figure 2E) and ICM was rarely found to be affected by zona opening except for one case, in which a large ICM was transiently trapped by opened zona opening. The empty zona after hatching following LZD exhibited a long and wide zona opening (Figure 2F). All human hatching blastocysts completely hatched following

Table I. Effects of long zona dissection (LZD) assisted hatching (AH), three-dimensional partial zona dissection (3D PZD) AH and control with no AH on development and hatching of 8-cell mouse embryos

<table>
<thead>
<tr>
<th>Method</th>
<th>No. of embryos</th>
<th>No. (%) of blastocysts</th>
<th>No. (%) of OPHB</th>
<th>No. (%) of complete hatching of blastocysts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day 4</td>
<td>Day 5</td>
</tr>
<tr>
<td>LZD 3D</td>
<td>180</td>
<td>165 (91.7)</td>
<td>165 (100)</td>
<td>12 (7.3)</td>
</tr>
<tr>
<td>PZD</td>
<td>161</td>
<td>142 (88.2)</td>
<td>135 (95.1)</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>130</td>
<td>118 (90.8)</td>
<td>107 (90.7)</td>
<td>0</td>
</tr>
<tr>
<td>( P^a )</td>
<td></td>
<td>&gt; 0.05</td>
<td>&lt; 0.05</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Rate of blastocyst is based on total number of embryos, but all other rates are based on the number of blastocysts.

\( ^a \)Pearson’s \( \chi^2 \)-test.

OPHB = observable progressing hatching blastocyst; refers to blastocysts with observable zona expanding or cells hatching out of zona.
Table II. Effect of different zona slit sizes on human blastocyst hatching

<table>
<thead>
<tr>
<th>State of blastocysts</th>
<th>LZD</th>
<th>MZD</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of blastocysts</td>
<td>20</td>
<td>39</td>
</tr>
<tr>
<td>No. of hatching blastocysts</td>
<td>20</td>
<td>39</td>
</tr>
<tr>
<td>No. (%) of blastocysts hatched out completely</td>
<td>20 (100)</td>
<td>18 (71.8)</td>
</tr>
<tr>
<td>ICM trapping and ICM size in MZD group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of large ICM trapping/no. of large ICM (%)</td>
<td>8/15 (53.3)</td>
<td></td>
</tr>
<tr>
<td>No. of small ICM trapping/no. of small ICM (%)</td>
<td>3/24 (12.5)</td>
<td></td>
</tr>
</tbody>
</table>

Values in parentheses are percentages.
*P < 0.05, Pearson’s χ²-test.
**P < 0.05, Fisher’s exact test, inner cell mass (ICM) with its volume larger than one-fifth of the volume of ZP during hatching is identified as big ICM, those smaller than one-fifth is identified as small ICM.
LZD = long zona dissection; MZD = moderate zona dissection.

Discussion
The slit for PZD or 3D PZD in human embryos is usually within 30–40 μm in length by single gap or crossed gap (Cieslak et al., 1999), and the hole of zona drilling by Tyrode’s acidic solution (Selva, 2000) as well as the zona hole from laser drilling (Blake et al., 2001) are usually within 20–40 μm in width. All these sizes range from one-fifth to one-quarter of embryonic diameter. We designed a usual/moderate size of zona opening on mouse embryos with two crossed slits of length 20–30 μm by 3D PZD as well as on human embryos with one slit of of 55–65 μm length by MZD. Our results indicate that zona state is an important factor affecting the opportunity for blastocysts to overcome the resistance of the zona in the hatching process. In the control group, blastocysts must build up pressure by expansion in order to thin the zona and complete hatching; however, in AH groups, blastocysts need only a little expanding pressure to protrude trophectoderm cells out of the zona, especially in the LZD group. Furthermore, our results also indicate that the size of zona opening during blastocyst hatching may be the key factor affecting the completion of the hatching process in vitro. Long zona slit following LZD with the size beyond two-thirds of zona diameter can be extended to a large zona opening by blastocyst expansion (Figure 2E and F), allowing 93.9% of mouse blastocysts and 100% of human blastocysts to achieve complete hatching in vitro, with blastocysts rarely trapped. However, 3D PZD or MZD methods produced small or moderate-sized zona opening in most cases, which squeezed the hatching blastocyst into an ‘8’ shape and often trapped large ICM (Figure 2D). In addition, the hatching speed also became very slow and a portion (33.1%, Table I) of them halted the hatching process.

During AH, the internal pressure generated by expanding blastocysts is released through the zona opening, and is too small to slit the zona opening to a larger size. So the size of the zona opening during hatching is basically determined by the original zona opening size created by AH. Thus, zona opening with originally usual/moderate size tends to cause blastocysts to fail complete hatching, especially in cases of good embryos with large ICM (Figure 2D, Table II). Despite years of investigation, the exact mechanism of zona escape in vivo has yet to be elucidated. If the embryos developed in vivo following AH, the ICM trapping would occur in the same way as in vitro. That would create a danger of the embryo being damaged or slit, which has been suspected as the cause of the monozygotic twinning following AH (Cohen et al., 1990; Alikani et al., 1994). So, a good AH method evaluated in vitro must produce a large enough zona opening during hatching and achieve a high rate of complete hatching of blastocysts, whereas zona opening with originally moderate or small size by AH may be defective in blastocyst hatching. Mantoudis et al. (2001) have reported that higher implantation rates can be obtained by zona thinning rather than zona opening, and further higher implantation rate can be obtained as the thinned zona area increases (Mantoudis et al., 2001). This improved outcome might be due to the larger zona opening during hatching in vivo, because the accumulated internal pressure of hatching the blastocyst may break through the thinned zona area and achieve a larger zona opening than the zona opening from AH, after which blastocysts cannot accumulate internal pressure during hatching.

However, zona thinning by chemicals (Cohen et al., 1992) or laser (Mantoudis et al., 2001) may pose potential harm to embryos. Furthermore, laser AH is too expensive for many IVF laboratories. PZD (Cohen et al., 1990) is a simple and economical method without the risk of chemical or optical harm to embryos inherent in the other two methods, but it is difficult to obtain a satisfactory size of zona opening (Cieslak et al., 1999; Balaban et al., 2002). Even though the 3D PZD (Cieslak et al., 1999) has improved the zona opening size by using two crossing PZD, the opening is still not large enough to achieve a high rate of complete hatching of blastocysts (Table I). Furthermore, in PZD or 3D PZD, mechanical harm, such as squeezing or injury to embryos, may occur during the manipulation, especially during ZP rubbing, while the hatching needle may slip from holding the pipette and strike the embryo on the holding pipette, causing a rapid mechanical pressure to the embryo. The mechanical change of hydrostatic pressure has potential harmfulness to spindle microtubules in living cells (Macas et al., 1996). Benefited by the use of the modified holding pipette with bevelled opening and the use of modified zona dissection procedure, the manipulation of LZD is stable and easy with no squeezing or buffeting the embryo, even using a hatching needle with a blunted tip. LZD therefore causes little or no distortion and injury to embryos, and is independent of the size of perivitelline space (PVS), as it can produce PVS by pushing embryonic cells aside under the ZP using a hatching needle with blunt tip. LZD can also apply to blastocysts for AH, even to the expanding blastocysts with no PVS under ZP, with no harm to blastocysts. However, it is difficult for other methods to achieve AH at blastocyst stage, since they all depend on the size of PVS to select for AH site, so as to reduce mechanical, chemical or optical damage to embryos during manipulation. So, compared with traditional PZD or 3D PZD, the LZD technique not only allows an easier
manipulation to create a markedly longer zona slit, which enhances the rate of complete hatching of blastocysts in vitro, but also decreases the potential risk of mechanical harm to embryos. However, the improved hatching rates and improved manipulation using LZD in vitro do not predict better implantation rates in vivo, and further investigation by clinical trials is needed to evaluate this new technique.

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References


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