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ASSISTED REPRODUCTION TECHNOLOGIES

Effect of the site of assisted hatching on vitrified-warmed blastocyst transfer cycles: a prospective randomized study

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Abstract
Purpose To assess the effect of assisted hatching (AH) site on the clinical outcomes in vitrified-warmed blastocyst transfer cycles.
Methods A total of 160 women who underwent vitrified-warmed blastocyst transfer cycles were randomized to either the ICM group (AH performing at the site near the inner cell mess, ICM), or the TE group (AH performing at the site opposite to the ICM). AH with laser zona drilling was performed 20 or 30 min after thawing once the ICM can be detected. Clinical pregnancy rate, implantation rate, live birth rate and the occurrence rate of monozygotic twins (MZT) pregnancy after transfer of these two groups were compared.
Results No significant difference was found in the clinical pregnancy rate (63.8 % vs. 67.5 %), implantation rate (51.7 % vs. 53.6 %) and live birth rate (57.5 % vs. 62.5 %) between the ICM group and the TE group. The occurrence rate of MZT was comparable between the two groups (3.9 % vs. 5.6 %).
Conclusions The site of assisted hatching has no influence on the implantation, pregnancy and live birth rate in human vitrified-warmed blastocyst transfer cycles.

Keywords Assisted hatching · Blastocyst · Vitrification · Pregnancy rate · Live birth rate

Capsule The site of laser assisted hatching, at either the embryonic or abembryonic pole, has no influence on the pregnancy rate and live birth rate in vitrified-warmed blastocyst transfer cycles.

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Introduction

The human early embryo is protected by a bilayered acellular matrix, the zona pellucida (ZP) [1]. It is essential for normal fertilization [2] and can maintain the integrity of the early embryo and protect the embryo from micro-organisms and immune cells before implantation [3]. But the embryo at the blastocyst stage must hatch from the ZP completely. It is a prerequisite for implantation.

The hatching difficulties are supposed to be one of reasons for implantation failure [4]. The artificial rupture of the zona pellucida is known as assisted hatching (AH) and it has been proposed as a method for improving the implantation capacity of the embryos [4, 5]. Although the clinical effect of AH in unselected patients is now still under debate, recent meta-analysis indicated AH was related to increased clinical pregnancy rate in women with frozen-thawed embryos [6, 7].

AH was performed in numerous previous studies of cryopreserved embryo transfers and a variety of AH techniques have been employed, including partial zona dissection, zona thinning, zona drilling, expanding the ZP or complete removal of the ZP, using mechanical, chemical techniques, laser, and piezon technology [see review 8]. However, there was no international standard protocol for AH technique. The size of the opening [9–11], the depth of the micro dissection in ZP [12, 13] and the site of AH [14] may all influence the effectiveness of AH. A recent in-vitro observational study of vitrified blastocysts suggested the existence of polarity in the hatching process of human embryos [14]. Artificial opening at the site close to the inner cell mass (ICM) resulted in higher rates of complete hatching. However, AH performing at the opposite site caused trapping of the ICM within the zona pellucida. Therefore, the authors suggested that the choice of AH site should be paid attention to because AH performed without consideration of the site may inhibit the hatching process.

However, the results of the in-vitro data may not reflect the behavior of blastocyst hatching in vivo. As far as we know, there are no reports concerning the possible influence of the AH site on the clinical outcomes after embryo transfer. The aim of this study was to assess the effect of AH site on the clinical pregnancy rate, implantation rate, live birth rate and the occurrence of monozygotic twins in vitrified-warmed blastocyst transfer cycles.

Materials and methods

Patient selection and assignment

Patients attending our Assisted Reproduction Unit for vitrified-warmed blastocyst transfers were recruited if they had one or two survival blastocysts after warming and agreed to receive AH. Some were excluded when: (1) patients were older than 40; (2) blastocysts of the patient developed to hatching stage or hatched before vitrification, and (3) there were air bubbles attached to the zona pellucida or there was a clear breach on the ZP after warming. From May 2011 to December 2011, 160 patients were enrolled. All patients gave written informed consent, and the study was approved by the university ethics committee of Tongji Hospital.

Just after warming, patients were randomized to either the ICM group (AH performing at the site near the ICM), or the TE group (AH performing at the site opposite to the ICM) according to a computer-generated table. Both patients and the clinicians were blinded to the group allocation. The procedure of AH was performed all by one specific embryologist.

IVF procedures

Ovarian stimulation was performed using follicle-stimulating hormone (Gonal-F, Serono, Sweden) with gonadotrophin-releasing hormone (GnRH) agonists using either long or short protocol. Women were administered human chorionic gonadotrophin (HCG, Profasi, Serono, Sweden) when dominant follicles were 18 mm diameter. Oocyte retrieval was performed 36 h after HCG administration using a vaginal ultrasound-guided procedure. Insemination or intracytoplasmic sperm injection (ICSI) were carried out 4–6 h after oocyte retrieval. The day of oocyte retrieval was considered as day 0. Fertilization was confirmed by the presence of two pronuclei 16–20 h after insemination/injection.

Embryo culture

Zygotes were cultured individually in droplet of G1 medium (Vitrolife Ltd, Gothenburg, Sweden) until day 3. Two embryos of top quality were transferred to the patient on day 3. The embryos with less than 4 blastomeres on day3, showed no cleavage on day 2 and (or) with >50 % fragmentation were discarded on day3 morning because they were supposed to have poor viability [15]. The surplus embryos were cultured in G2 medium (Vitrolife Ltd, Gothenburg, Sweden) under 5 % O_2, 6 % CO_2, and 89 % N_2 until day 6. If the number of retrieved oocyte exceeded 20 and there was high risk of ovarian hyperstimulation syndrome (OHSS), 4 embryos with good quality were vitrified on day3 and the surplus embryos were extended cultured as mentioned above.

Embryos were checked on the morning of day 5 and day 6 to observe the formation of blastocyst. Blastocyst quality was assessed according to Gardner's scoring system [16]. The blastocyst was first scored from 1 to 6 according to the diameter of the blastocyst and the expanding status. The ICM was scored as follows: A: tightly packed, many cells: B: loosely grouped, several cells; C: very few cells. The trophectoderm grading was as follows: A: many cells forming a cohesive epithelium: B: fewer cells forming a loose epitheliums; C: very few large cells. The good quality blastocysts scoring B or higher for both ICM and trophectoderm grades (≥3BB) were vitrified.

Vitrification of blastocysts

Blastocysts were vitrified according to the protocol developed by Kuwayama et al. [17] using a cryotop (Kitazato Supply Co. Fujinomiya, Japan). Before the beginning of vitrification, blastocysts were artificially shrunk using a 1,480 nm noncontact laser system (OCTAX EyeWare, Germany) as described by Mukaida et al. [18]. The ICM was located away from the targeted point of the laser pulse. One or two laser pulse(s) (2.0 ms) targeted at the cellular junction of the trophectoderm was (were) performed to induce collapsing of the blastocoelic cavity. The blastocysts were usually shrunk in 10 min. The procedures of vitrification were performed at room temperature (22–26 °C). The shrunk blastocysts were firstly equilibrated in 1 ml equilibration solution for 5 min, then transferred to 1 ml vitrification solution and loaded onto the cryotop within 1 min. Then the cryotop was submerged into liquid nitrogen immediately and then protected by the plastic cover.

Warming of blastocysts

The plastic cover was removed in liquid nitrogen and the end of the cryotop was immersed directly into 1 ml of 37 °C TS (1.0 mol/l sucrose solution) for 1 min. The blastocysts were then transferred into 1 ml of DS (0.5 mol/l sucrose solution) for 3 min at room temperature and washed twice in the base medium for 5 min at room temperature. After

warming, the blastocysts were rinsed several times and cultured in fresh G2 droplet at 37 °C.

Assisted hatching and embryo transfer

Blastocysts were assessed by an inverted microscopy at 0, 10, 20 and 30 min after thawing for observation of the re-expansion of the blastocysts. The blastocysts were usually re-expanded partially within 20 min after warming. AH with laser zona drilling (50 μm) was performed 20 or 30 min after thawing once the ICM can be detected. The blastocysts were positioned with the laser target located at the site near or opposite to the ICM. A single laser shot created a hole of 10 μm in the ZP. Several laser pluses were applied to make a 50 μm opening of the ZP. It took about 2 min for each blastocyst.

The blastocysts were then rinsed and transferred to G2 droplet and cultured under 5 % O_2, 6%CO_2, and 89 % N_2. The blastocysts were observed 2 h after warming by an inverted microscopy and re-expanded blastocysts were judged to be survived. Embryo transfer was performed 2–3 h after warming by the guidance of abdominal ultrasound.

Endometrial preparation and assessment of pregnancy

Vitrified-warmed blastocyst transfer was performed in either hormonal replacement treatment cycles or naturally cycles. In hormonal replacement treatment cycles, patients received estrogen for 12 days from the beginning of the menstruation and then were administrated progesterone when endometrial thickness exceeded 8 mm. Embryo transfer was scheduled on day 5 after the initiation of progesterone treatment. In natural cycles, embryo transfer was scheduled on 5 days after ovulation.

Serum HCG was measured 2 weeks later and a clinical pregnancy was confirmed when a fetal heart beat was identified via ultrasound 4 weeks after transfer. Monozygotic twins' pregnancy was identified when there were two heart beats in one gestational sac or the number of gestational sacs exceeded that of transferred blastocysts.

Statistical analysis

Statistical differences between groups were analyzed by Student's *t*-test and Chi-squared test as appropriate. Statistical significance was defined as a P-value<0.05.

Results

During the study period, 579 vitrified-warmed blastocyst transfer cycles were approached. In total, 160 couples enrolled undergoing 160 vitrified-warmed blastocyst transfer cycles were randomly allocated into two groups: 80 cycles in the ICM group and 80 cycles in the TE group.

Age of the woman at vitrification and warming, duration of infertility, the percentage of primary infertility, the cause of infertility, the insemination method, the percentage of cancelled embryo transfer in fresh cycles, the number of retrieved oocytes and vitrified blastocysts in stimulated cycles were showed in Table 1. There were no significant differences in these parameters between the two groups (Table 1).

The type of endometrial preparation, the thickness of the endometrium, the percentage of day5 and day6 blastocysts, the survival rate after warming were similar between the two groups. The mean number of transferred blastocysts, the expanding status before vitrification, the ICM score and the TE score of the transferred blastocysts were also comparable. (Table 2)

After embryos transfer, no difference was found in implantation rate (51.7 % vs. 53.6 %) and clinical pregnancy

Table 1 Patient characteristics in the last fresh cycle for the two groups of assisted hatching

	ICM group	TE group
No. of cycles	80	80
IVF cycles/ICSI cycles	56/24	55/25
Mean age of women at vitrification (years) ± SD	29.8±3.2	29.6±4.1
Mean age of women at warming (years) ± SD	30.3±3.3	30.1±4.1
Duration of infertility (yr) (mean ± SD)	4.5±2.9	4.4±3.2
Primary infertility/secondary infertility	43/37	44/36
Tubal factor	51	49
Endometriosis	8	9
Male factor	17	19
Unexplained	4	3
No. of cycles cancelled embryo transfer due to OHSS	42	41
No. of retrieved oocytes (mean ± SD)	20.3±8.1	20.3±8.9
No. of blastocysts vitrified (mean ± SD)	3.7±2.0	3.9±2.7

There were no statistically significant differences between the two groups

OHSS ovarian hyperstimulation syndrome

ICM group: 50 μm opening of the zona pellucida by laser assisted hatching at embryonic pole; TE group: 50 μm opening of the zona pellucida by laser assisted hatching at abembryonic pole

Table 2 Comparison of the characteristics of warming cycles and morphology of blastocysts between the two groups of assisted hatching

	ICM group	TE group
Type of endometrial preparation (Natural cycle/HRT cycle)	40/40	33/47
Thickness of the endometrium (mm)	9.0±1.4	9.2±1.5
No. of cycles transferred with Day5/D6 blastocysts	45/35	41/39
No. of blastocysts warmed	147	139
No. of blastocysts survived (%)	143 (97.3)	138 (99.3)
Mean no. of blastocysts transferred±SD	1.8±0.4	1.7±0.5
Expanding status of blastocysts before vitrification		
3	34	29
4	109	109
ICM score		
A	44	40
B	99	98
TE score		
A	34	39
B	109	99

There were no statistically significant differences between the two groups

HRT cycle hormonal replacement treatment cycle

rate (63.8 % vs. 67.5 %) between the ICM group and the TE group. The pregnancy rates of day5 and day6 blastocysts were comparable in both the ICM group (64.4 % vs. 62.9 %) and the TE group (68.3 % vs. 66.7 %). (Table 3)

There were two and three triplet pregnancies and were diagnosed as monozygotic twin (MZT) pregnancies in ICM group and TE group separately; the occurrence rate of MZT was comparable between the two groups (3.9 % vs. 5.6 %). All the women who got triplet pregnancies accepted selective fetal reduction by transvaginal embryo aspiration according to the Rule of Assisted Reproductive Technology in China and all of them had delivered babies.

In total, 46 and 50 women gave birth to 65 and 64 live infants after 28 weeks of gestation separately in ICM group and TE group patients. The live birth rate (57.5 % vs. 62.5 %) was comparable between the two groups. All infants have had normal physical profiles (Table 3).

Discussion

This study was set up to assess the effect of assisted hatching site on the clinical outcomes in vitrified-warmed blastocyst transfer cycles. Results of this study suggested that the site of assisted hatching with laser drilling, either near or opposite to the ICM, had no influence on the implantation, clinical pregnancy and live birth rates after transfer of vitrified blastocysts.

The results of our study were not in line with a previous in-vitro observational study [14] and the proposed theory about the polarity of hatching process. A study of Miyata H [14] showed that AH of vitrified blastocysts at the site close to the ICM resulted in higher rate of complete hatching (68.8 %, 11/16) after 48 h in vitro culture. However, only 12.5 % (2/16) blastocysts hatched completely after AH at the abembryonic pole. The in vitro study suggested AH at the site close to the

Table 3 Comparison of the clinical outcomes of vitrified blastocysts transfer cycles between the two groups of assisted hatching

	ICM group	TE group
No. of blastocysts transferred	143	138
No. of embryos implanted (%)	74 (51.7)	74 (53.6)
No. of clinical pregnancies (%)	51 (63.8)	54 (67.5)
Singleton	27	34
Twin	21	17
Triplet	2	3
Ectopic	1	0
No. of clinical pregnancies of day5 blastocysts (%)	29 (64.4)	28 (68.3)
No. of clinical pregnancies of day6 blastocysts (%)	22 (62.9)	26 (66.7)
No. of MZT pregnancy (%)	2 (3.9)	3 (5.6)
No. of live birth (%)	46 (57.5)	50 (62.5)
No. of babies (male: female ratio)	65 (38:27)	64 (41:23)

There were no statistically significant differences between the two groups

MZT monozygotic twins

ICM might have better outcomes than AH at abembryonic pole. However, results of the present study showed that the clinical outcomes were comparable after transferring blastocysts with AH at different sites. Several points may explain this discrepancy.

Firstly, the hatching process in vivo differs from that of in vitro. In addition to the increased internal pressure, "protease" with zonalysis activity from the trophectoderm and (or) the uterus [19–21], repeated collapses and expansions of the blastocyst [22] and sphincter-like zona-breaker cells [23] are involved in the hatching process. Data from hamster and mouse showed that successful hatching in vitro was dependent on a sufficiently high number of embryonic cells, which enables blastocyst expansion and zona shedding. The zona was focally lysed; then blastocysts extruded through the hole, leaving most of the zona intact [24, 25]. However, the lower number of embryonic cells detected in zona-free blastocysts in vivo, indicated the mechanism of zona escape in vivo did not depend on blastocyst expansion [25]. The zona was globally lysed and gradually thinned. Retarded embryos also showed global zona lysis in vivo, implying that intrauterine zona lytic activity occurs during the peri-implantation stage [24, 26]. These results implicate a uterine contribution to normal zona escape in vivo that is lacking in vitro. Therefore, it is not surprising that AH at different sites had no influence on the clinical outcomes after vitrified-warmed blastocysts transfer.

Secondly, the size of zona opening probably affects the results. In the study of Miyata H [14], a small opening of 20 μm was performed. When AH was performed at the embryonic pole, the ICM exited at first and the blastocyst hatched easily. In contrast, when the small TE cells extruded at first from the hole at the abembryonic pole, the ICM was trapped in the zona, resulting in a lower rate of complete hatching. In the present study, the AH opening was enlarged to 50 μm, with less possibility of the ICM trapping. An in vitro study in our center showed that the complete hatching rate was 57.9 % (11/19) in vitrified-warmed blastocysts after AH (50 μm opening) at the abembryonic pole (unpublished data), which was significantly higher than the result of the previous study [14]. This indicated the size of AH opening can influence the hatching process.

Furthermore, hatching process still remains one of the least studied phenomenons of early embryo development and previous observational studies about the spontaneous hatching site of human embryos were controversial. Some studies demonstrated that natural hatching sites of human blastocysts were usually close to the ICM [22, 27], but others found the human embryos hatched from the abembryonic pole in most cases and rarely at the ICM pole through video observation and critical examination of 30 human blastocysts by transmission electron microscopy [23, 28]. Study of blastocyst transfer in fresh cycles also demonstrated that only 38.9 % of all the hatching blastocysts showed a zona breach close to the ICM [29].

One report of fresh blastocyst transfer cycles [30] found that the clinical pregnancy rate of cycles with transferred blastocysts that hatched from embryonic pole was higher than that of cycles with the blastocysts herniated from the abembryonic pole (72 % vs. 51 %, $P<0.05$). The author proposed hatching close to the ICM would accelerate contact between blastocyst and uterus, especially for those trophectodermal cells supposed to draw the blastocyst into the uterine wall. This mutual interaction between blastocyst and uterus may be impaired or delayed if hatching takes place opposite the ICM. The shift in the window of receptivity with 1–2 days earlier in stimulated cycles [31] may enhance the effect of hatching speed (site) on implantation outcomes. In the present study, the better synchronization between embryo and endometrial in frozen embryo transfer cycles versus stimulated cycles may weaken the difference of hatching speed (site) and result in comparable outcomes.

There were two breaches in the zona of the ICM group blastocysts (one hole of 10 μm at the site far from the ICM before vitrification and one AH opening of 50 μm after warming). However, we proposed the hole resulted from laser collapsing had no influence on the clinical outcomes. Theoretically, blastocysts may hatch from the AH hole because the AH opening was much larger and the viscosity at the AH site during the hatching process should be lower than that of the first hole. The hatching process of blastocysts after AH at the site near the ICM was observed in an in vitro study in our center. Laser collapse and AH were performed as described in the present study. All blastocysts (20/20) showed initiation of hatching and extrusion of the ICM at the AH site and 90 % (18/20) blastocysts showed complete hatching 36 h after warming. This result indicated the existence of the first breach at the abembryonic pole did not result in stuck of the ICM or hinder the hatching process.

The occurrence of MZT was supposed to be one of the potential risks of AH [32]. In the present study, the occurrence rate of MZT was comparable between the ICM group and the TE group (3.9 % vs. 5.6 %). Both of them were higher than that of the vitrified blastocyst transfer cycles without AH during the study period (3/205, 1.5 %) and previous AH groups (2/107, 1.9 %) that were performed in our center (opening with the same size at the site with largest perivitelline space, immediately after warming). However, the difference was not statistically significant.

The high rate of monozygotic twins in the present study may be related to several factors as follows:

(1) More frequency of observation and operation in vitro, longer expose time out of the incubator may have potential influence to the embryos' development. (2) Artificial shrinkage before vitrification and AH after warming were performed by laser. The proximity to the embryo and the

application of multiple shots may pose potential harm to the blastocyst. (3) The laser-ablated hole was much 'stiffer' than the openings created with acidic Tyrode's solution [33]. The rigidity of the zona may affect the hatching process. (4) The opening of 50 μm probably is not large enough for the hatching process. Large ZP opening (1/2 or 2/3 of the zona diameter) [10, 34] or total removal of the ZP [35] resulted in higher delivery rates than partial opening. Whether the possibility of MZT can be reduced by enlarging the opening size or total removal of the ZP needs more clinical data.

In conclusion, this study suggested that the assist hatching site of vitrified-warmed blastocysts had no influence on the implantation, pregnancy and live birth rate in human vitrified blastocyst transfer cycles. Further studies are needed to ascertain the theory about hatching polarity and assess the effectiveness of assisted hatching by choosing an appropriate site or making opening with larger size.

Disclosure None.

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