A study comparing three different laser-assisted hatching techniques

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Summary

Purpose of investigation: Laser-assisted hatching (LAH) is recognized as a useful technology to improve clinical pregnancy rates and implantation rates. This study reports the differences between a new LAH method and two conventional LAH techniques. *Materials and Methods:* The authors studied 151 patients with repeated implantation failure, who were divided into three groups. *Results:* In group 1, the zona pellucida (ZP) was opened using LAH (n = 52). In group 2, laser-assisted thinning was performed to dissolve the outer layer of the ZP (n = 49). In group 3, laser-assisted thinning was performed to dissolve the inner layer of the ZP (n = 50). The clinical pregnancy rates and implantation rates among the groups were compared. The results demonstrate that there are significant differences in the clinical pregnancy rates and implantation rates between group 3 and the other two groups. *Conclusion:* Performing laser-assisted thinning to dissolve the inner layer of the ZP markedly increases the pregnancy rates and implantation rates of patients with repeated implantation failure.

Key words: Laser-assisted hatching; Recurrent implantation failure; Clinical pregnancy rates; Implantation rates.

Introduction

Assisted hatching (AH) has been shown to effectively increase implantation and pregnancy rates, especially in patients with advanced ages [1-5], high follicle stimulating hormone (FSH) levels [6, 7], recurrent implantation failure [8-13], or cryopreservation [14-18]. AH methods include mechanical partial zona dissection, chemical zona drilling, and laser techniques. Studies comparing these methods have reported that laser-assisted hatching (LAH) is more beneficial to the embryo than other AH techniques [19, 20] and that LAH is a safe and rapid method [21, 22].

The zona pellucida (ZP) of human embryos is bilayered; the outer layer is thick and easily dissolved, whereas the inner layer is more compact, resilient, and difficult to dissolve [23]. Physiologically, upon reaching the blastocyst stage, the human embryo hatches from the ZP and has a full communication with the endometrium. Precipitous communication between the cleaved embryo and endometrium may be disadvantageous to the embryo. Conventional ZP thinning of a day-3 embryo may not dissolve the inner layer, which is the main barrier to hatching. Advances in laser technology make it possible to remove the inner layer of the ZP. According to the ZILOS-tk manual [24] and physics, it is impossible to create a hole through the entire zona with the new ZP thinning method, which removes the inner layer of the ZP.

The objective of the present study was to evaluate the outcomes of fresh day-3 embryo transfer after AH with three types of LAH: ZP opening, conventional ZP thinning, and new ZP thinning (removing the inner layer of the ZP), in women with recurrent assisted reproductive technology (ART) treatment failures.

Materials and Methods

Study period and patients

A total of 151 LAH patients took part in the study from November 2009 to August 2010 at the Reproductive Center of the Shenzhen Police Hospital, a public hospital in Shenzhen. All patients gave a written informed consent to participate in this study, and the study design was approved by the local institutional ethics committee. All eligible patients had at least three previous implantation failures in fresh day- 3 embryo transfers. Furthermore, in order to minimize the contribution of age as the risk factor for implantation failure, the patients included in the study were 37-years-old and younger. AH was explained to all of the patients who took part in the study before the embryo transfer. On the day of embryo transfer, the patients were randomly selected, beginning with the ZP opening group (group 1, 52 patients), the conventional ZP thinning group (group 2, 49 patients), and the new ZP thinning group (group 3, 50 patients). This protocol was repeated every other day throughout the study. In group 1, 40 μm defect was made in ZP.

Patient treatment

The women were treated with the gonadotropin-releasing hormone analogue triptorelin acetate from either the preceding mid-luteal phase in a long treatment protocol or the second day of the cycle in a short treatment protocol. Ovarian stimulation was carried out with human menopausal gonadotropin (hMG) or recombinant human FSH. Follicular development was monitored with serial vaginal ultrasound examinations and serum estradiol (E2) measurements. Human chorionic gonadotropin (hCG; 10000 IU) was administered intramuscularly (i.m.) when the dominant follicles reached 18 mm in diameter and at least

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two follicles were ≥ 17 mm in diameter. Oocytes were retrieved with transvaginal ultrasonographic guidance 35 h after injection of hCG. The luteal support was initiated on day 1 after oocyte retrieval with 60 mg/day of progesterone (Xianju, Taizhou, China), which was administered until the measurement of serum beta-hCG on day 14 and prolonged until 12 weeks in cases of pregnancy. In group 1, 29 patients had three cycles, 12 patients had four cycles, eight patients had five cycles, and three patients had six cycles of their in vitro fertilization (IVF) embryo transfers. In group 2, 30 patients had three cycles, 13 patients had four cycles, four patients had five cycles, one patient had six cycles, and one patient had seven cycles of their IVF embryo transfers. In group 3, 33 patients had three cycles, ten patients had four cycles, five patients had five cycles, and two patients had six cycles of their IVF embryo transfers.

In vitro fertilization procedure

After retrieval, all oocytes were incubated in 0.6 ml Quinn's Advantage Fertilization Medium in four-well multidish with three to four oocytes per well. The oocytes were incubated in an atmosphere of six percent CO_2 at 37°C. Insemination was performed by introducing 500,000 motile sperm into each well, which contained the oocytes in medium under tissue culture oil. The multidish was then incubated overnight

Intracytoplasmic sperm injection (ICSI) procedure

After being denuded of the surrounding cumulus cells, the oocytes were incubated in a 50-mm plastic dish under tissue culture oil until the ICSI. ICSI was carried out only on oocytes that had extruded their first polar bodies.

Embryo culture and scoring

After checking for fertilization, each pronuclear stage zygote was cultured in a microdrop (30 µl) of Quinn's Advantage Cleavage Medium containing 10% Quinn's Advantage Serum Protein Substitute (SPS) in a 30-mm plastic dish until the third day. The day-3 embryos were assigned a numerical grade using the following scale: grade 1, fragmentation less than five percent with equally sized homogenous blastomeres; grade 2, five to 20% fragmentation with equally sized homogenous blastomeres; grade 3, 20%-50% fragmentation with unequally sized blastomeres; grade 4, over 50% fragmentation with unequally sized blastomeres. No more than three embryos in high quality (grades 1 or 2) were transferred to each patient. The remaining high-quality embryos were cryopreserved. Embryos unsuitable for cryopreservation (grades 3 or 4) were discarded.

AH procedures

AH was performed directly on the day-3 cleavage-stage embryos in the 30-mm dish. The embryos were kept in their original culture medium, and all the selected embryos were hatched using the ZILOS-tk laser (1.48-µm diode laser). To perform the procedure, the 30-mm dish was first placed on the stage of the microscope. Then under the 40 × laser-grade objective lens, the embryo was positioned so that a portion of the zona was in the path of the laser beam. The laser beam was activated using a pulse duration of 600 µs and 300 mW of power, and the laser was fired to create a hole in the zona.

In group 1, the embryos underwent laser zona ablation using several pulses, depending on the thickness of the zona pellucida. The final size of the hole made in the zona was measured to be 40 µm (Figure 1a). In the conventional ZP thinning group, multiple irradiations along the convex periphery of the ZP from outward to inward were used to thin 60%-80% of the ZP, and create a defect involving approximately 25% of the ZP circumference (Figure 1b). In group 3, the laser was used to remove the inner layer of the ZP. This procedure was performed from inward to outward, and approximately 40% of ZP thickness was ablated, creating a defect that involved approximately one sixth to one fifth of the ZP circumference (Figure 1c).

Table 1. — *Pathologic findings.*

	Group 1	Group 2	Group 3
Patient age (years)	34.2 ± 2.3	33.7 ± 2.7	34.4 ± 2.1
No. of embryo transfer cycles	52	49	50
No. of previous attempts	2.5 ± 0.4	2.4 ± 0.7	2.5 ± 0.5
Zona thickness (µm)	16.9 ± 2.4	16.1 ± 2.1	16.3 ± 1.9
Duration of infertility (years)	4.3 ± 2.1	4.1 ± 2.5	4.5 ± 2.0
No. of retrieved oocytes	9.7 ± 6.2	9.4 ± 6.9	9.1 ± 5.4
No. of two pronucleate (2PN)	6.7 ± 1.9	6.2 ± 2.4	6.6 ± 2.1
No. of embryos transferred	2.1 ± 0.5	2.2 ± 0.2	2.4 ± 0.3
No. of blastomeres in ET embryos	7.5 ± 0.6	7.7 ± 0.5	7.6 ± 0.5
Implantation rate	10.4%	11.2%	17.6%[a(3-1), a(3-2)]
Clinical pregnancy rate	21.2%	24.5%	32%[b(3-1), b(3-2)]
Multiple gestation rate	11.5%	12.2%	20%[c(3-1), c(3-2)]

[a(3-1)] $p = 0.022$; [a(3-2)] $p = 0.026$; [b(3-1)] $p = 0.02$; [b(3-2)] $p = 0.027$; [c(3-1)] $p = 0.023$ [c(3-2)] $p = 0.03$.

Statistical analysis

The Mann-Whitney test, unpaired Student's t test, χ^2 test, and the Fisher's exact test were used as appropriate to determine the statistical differences among the groups. A p value of < 0.05 was considered significant.

Results

Table 1 compares the data of the three study groups. There were no significant differences in the number of cycle attempts, zona thickness, mean age of the patients, duration of infertility, and the number of embryos transferred among the three groups. The implantation rate (IR) in group 3 was significantly higher than in group 1 (group 3 vs group 1: 17.6% vs 10.4%, $p = 0.022$) and group 2 (group 3 vs group 2: 17.6% vs 11.2%, $p = 0.026$). There were significant differences in the clinical pregnancy rates (PR) among the groups (group 3 vs group 1: 30% vs 21.2%, $p = 0.02$; group 3 vs group2: 30% vs 24.5%, $p = 0.027$). Significant differences were found in the multiple gestation rate (group 3 vs group 1: 20% vs 11.5%, $p = 0.023$; group 3 vs group 2: 20% vs 12.2%, $p = 0.03$).

Discussion

The possible reasons for repeated implantation failure in infertile patients include zona hardening, asynchrony between the embryo and the endometrial implantation window after ovarian stimulation, and deficiency in the cellular energy required for hatching [25]. Retrospective studies have suggested that AH is beneficial for patients with repeated previous implantation failures [11-13].

LAH is considered less traumatic than chemical- or mechanical-assisted hatching [26-28]. A previous study

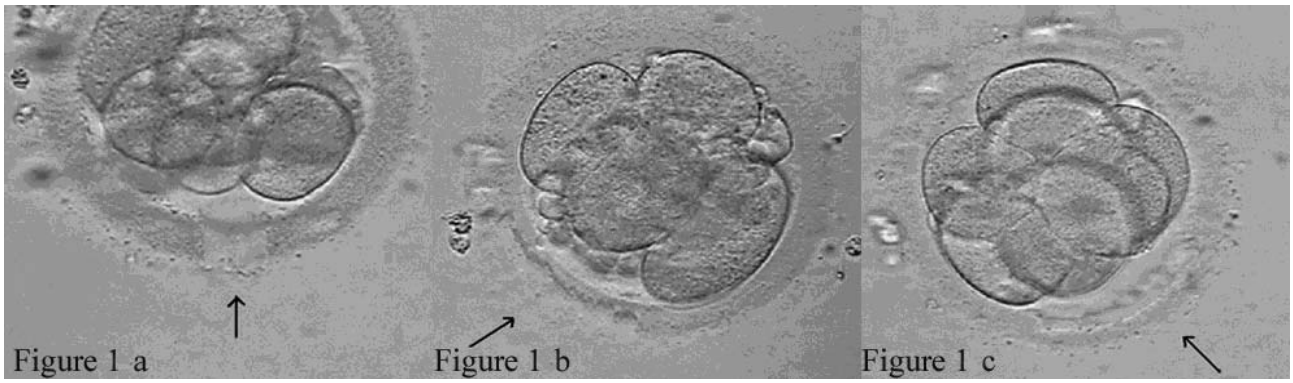

Figure 1. — Photomicrograph of a day-3 human embryo after zona opening with the laser. a) The opening in the zona pellucida is indicated by an arrow (magnification: × 400). b) The thinned area of the outer layer of the zona pellucida is indicated by an arrow (magnification: × 400). c) The thinned area of the inner layer of the zona pellucida is indicated by an arrow (magnification: × 400).

showed that AH does not increase the abortion rate or the number of biochemical pregnancies [29]. Furthermore, no increases in the major congenital malformation rate or the rate of chromosomal aberrations in children born after LAH [30, 31], confirmed the safety of this technique. Moreover, LAH is a rapid and convenient technique that can be completed in minutes.

Studies have also reported disadvantages of ZP opening and thinning. One study showed that ZP thinning alone was not effective in promoting implantation, due to the intact, hard, inner layer of the ZP [23]. ZP opening may increase the risk of bacterial infection of the embryo, as well as other harmful interactions with the environment. Moreover, the blastomeres could be more likely to separate during the cleavage of the embryo. Some researchers have reported that ZP opening could be more effective in AH of embryos than the ZP thinning [32, 33]. However, there is no consensus regarding the possible advantage of breaching or thinning the ZP in order to increase the IR and PR.

In light of the current debate and previous inconsistent study results, the new ZP thinning technology might provide the most effective way to aid embryo hatching from the ZP. This new method dissolves the inner layer of the ZP, which is regarded as the main barrier of hatching. Meanwhile, the outer layer is easily dissolved, and it still surrounds the blastomeres and protects the embryo from harmful interactions with the environment. The results of the present study demonstrate this point. The IR and PR of group 3 were significant higher than those measured in groups 1 and 2. Since the patients selected were randomized, patient age, ZP thickness, duration of infertility, embryo quality, and other factors did not affect the final results.

In the ZP opening group, the present IR results were similar to those in a previous study by Valojerdi *et al.* [34]; however, the PR was lower (21.2% vs 27.1%). One possible reason for this difference might be the difference in the target patients. In the present study, the patients included in the study had at least three previous implantation failures, and Valojerdi *et al.* defined recurrent implantation failures as ≥ two previous cycles. In the conventional ZP thinning group, the present results were similar to previous findings [12].

For a small percentage (6.4%) of embryos, the entire internal surface is close to the blastomeres with no gap between the internal surface and the blastomeres. In these cases, the new ZP thinning method may cause more thermal damage to the blastomeres than other LAH techniques, since the dissolving points are very close to the blastomeres. A further long-term study will be needed to determine whether this damage is serious. The authors found no similar study reported by other researchers.

It should be noted that this study is limited by its small sample size (power = 0.6). Hence, it is very possible that biologically significant differences would not have emerged as statistically significant due to inadequate power. Despite this limitation, the authors obtained good results with this new LAH method in group 3. A follow-up study with more subjects should be pursued to confirm the present pattern of results.

In conclusion, the authors report the successful use of a new LAH technique for ART treatment. The data indicated that the new ZP thinning technology is the most effective method to benefit patients with repeated implantation failures (≥ three previous implantation failures).

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