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Background: For infertile women aged over 37 years, failure of the zona pellucida to rupture is believed to be associated with a decreased implantation rate in in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI). It has been suggested that assisted hatching of embryos will increase the pregnancy rate with IVF by means of mechanically facilitating the hatching process.

Methods: One hundred twenty cycles of IVF/ICSI in females aged over 37 years were analyzed. Women included in this study were allocated into 2 groups. In group I, embryos were cultured and transferred without laser-assisted hatching (LAH), whereas embryos of group II were examined and treated with LAH just before being transferred. Laser manipulations were performed using a 1.48-µm (infrared) diode laser (Fertilase). The laser was aimed at the zona to create openings of about 20 µm in diameter.

Results: The mean ages of women in groups I and II were 38.8 ± 1.8 and 39.5 ± 1.6 years, respectively (p = 0.17). The number of retrieved oocytes, endometrial thickness, and number and quality of transferred embryos did not significantly differ between the 2 groups. Rates of implantation (7.3% and 6.7%, respectively, p = 0.89), pregnancy (16.3% and 17.5%, respectively, p = 0.86), and early pregnancy loss did not differ between the 2 groups.

Conclusion: Our data failed to demonstrate any benefit of LAH in improving implantation or pregnancy rates in women of advanced age, suggesting that factors other than laser drilling of the zona should be considered.

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Key words: diode laser, assisted hatching, advanced female age.

Even with the progression of assisted reproductive technologies (ARTs), pregnancy and implantation rates are still low. Failure of zona pellucida (ZP) rupture and the subsequent impaired escape of embryos from the ZP have been proposed to partially account for the decreased implantation rate in ARTs. It has been suggested that assisted hatching (AH) of embryos would enhance implantation by means of mechanically facilitating the hatching process and the subsequent interaction of embryos.

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with the endometrium. AH of cleaved embryos to increase implantation and pregnancy rates has been reported by several authors. With AH, enhancement of implantation increases with age, being more prominent after the age of 38. In recent research, there is evidence showing benefits of AH. The implantation rate increased in women aged 35-39 years and markedly increased in women aged 40-42 years without increasing the incidence of spontaneous abortions or of monozygotic twins. Pregnancy after in vitro fertilization and embryo transfer (IVF/ET) with AH in patients older than 38 years was higher compared to that in patients who did not undergo AH. However, others failed to show any benefit of AH, as no significant increase in the rates of implantation, pregnancy, or take-home babies was obtained by AH in women of advanced age.

Lasers have been safely used in AH, and the thermal effect can be minimized by taking certain precautions. Diode lasers (lambda of 1.48 µm) are used with in vitro fertilization to dissect the zona pellucida of pre-embryos. A focused laser beam is applied in vitro to form a channel or trench in the zona pellucida. The procedure is used to facilitate biopsies or to promote embryo hatching. Compared with partial zonal dissection (PZD) and acid Tyrode's solution, a laser is more efficient in assisting hatching. In this study, we used laser-assisted hatching (LAH) and attempted to demonstrate the effect of LAH on pregnancy outcomes in women of advanced age who received IVF/ET.

**METHODS**

**Subjects**

From October 1, 2000 to September 30, 2001, women aged 37 years or older undergoing IVF or intracytoplasmic sperm injection (ICSI) were included in the study. The criteria for selection were based on our data (unpublished) that the outcomes of IVF were poor in women older that 37 years. Women who fit the criteria were allocated into 2 groups according to the last digit of their chart number: patients with an odd number were allocated to group I, and those with an even number to group II. In group I (N=80), embryos were cultured and transferred without LAH, whereas embryos of group II (N=40) were examined and treated with LAH just before being transferred. One hundred twenty cycles were eligible. Among the infertile couples, 16 in group I and 13 in the group II underwent ICSI due to male factors (WHO 1993 criteria). Those women with poor ovarian reserves (elevated baseline follicle-stimulating hormone (FSH), e.g., day 3 FSH > 15 mIU/ml) and non-obstructive azoospermia were excluded.

**IVF and ICSI**

Ovarian hyperstimulation was induced by recombinant FSH (Gonal-F; Serono, Aubonne, Switzerland), human menopausal gonadotrophin (Pergonal; Serono), or a combination of both, with pretreatment using down-regulation by a gonadotropin-releasing hormone agonist (GnRH-a, leuprolide acetate, Lupron; Abbott, Chicago, IL). The growth and development of follicles were monitored using serum estradiol levels as determined by radioimmunoassay (RIA) and transvaginal ultrasound folliculometry. Human chorionic gonadotropin (HCG) 10,000 IU (Pregnyl; Organon, Oss, Holland) was not administered intramuscularly until at least 2 follicles greater than 18 mm in diameter were observed. Oocyte retrieval was performed under transvaginal ultrasound guidance 34-36 hours after HCG administration.

The procedure of IVF or ICSI was similar to that described in our previous study. Briefly, mature oocytes were examined and inseminated about 6-8 hours after retrieval. For ICSI, sperm was collected from ejaculate semen or from the epididymis (micro-epididymal sperm aspiration [MESA]). To avoid injury to the spindle, oocytes were held in such a way to keep the polar bodies in a 6 or 12 o’clock position. Ejaculate sperm was washed in a 15-ml conical tube (3215, Costar, Cambridge, MA) with human tubal fluid (HTF) medium containing 10% preovulatory serum. Epididymal specimens were placed in 500 µl of HTF-HEPES (Sigma Chemical, St. Louis, MO) after MESA. Oocytes were inseminated after sperm preparation and incubated at 37°C in a 5% CO₂ atmosphere. Embryos were examined the following morning with a change of media. The status of embryo growth was recorded. Embryo transfer was performed about 72 hours
after retrieval. Up to 6 grade 1-3 pre-embryos were transferred. Embryos with >50% fragmentation were not transferred. Embryo quality was graded using the system described by Veeck(20) before transfer. Pregnancy was detected with a urinary pregnancy test, and results that were negative or equivocal were further confirmed by RIA for serum β-HCG levels. Clinical pregnancy was defined as a distinct intrauterine gestational sac seen on transvaginal ultrasound.

**Laser-assisted hatching (LAH)**

Before transfer, embryos were examined, followed by LAH. Due to the specific wavelength, laser drilling was performed directly on embryos in the culture dishes. The dish was placed onto the displacement stage of an inverted microscope (Diaphot 300; Nikon, Tokyo, Japan), connected to a 1.48-µm (infrared) diode laser (Fertilase, Medical Technologies, Montreux SA, Switzerland); a 670-µm pilot light and a video monitor were used for visualization and for aiming at the target embryos. The setup used for zona drilling was similar to that described elsewhere.(17) For laser drilling, culture dishes with 8-cell-stage embryos were placed on the microscope stage under the 40X microscope objective. A tangential position of the zona pellucida of the embryos was focused on, and laser treatment was achieved using 1 or 2 laser irradiations at an average power of 45 mW for 20 to 25 ms. Openings of about 20 µm were created through the zona.(21) The zona at the site of the openings was totally ablated.

**Statistical analysis**

Pregnancy and implantation rates were analyzed on the basis of transfer cycles. In order to explore the influence of LAH, the pregnancy rate was analyzed by χ² test or Fischer's exact test, and other variables were examined using Student’s t-test. In all cases, p<0.05 was considered statistically significant.

**RESULTS**

One hundred twenty cycles were included in this study. The ages of female patients ranged from 37 to 45 (mean, 39.0; 2.2) years. Investigation of female factors showed that 35 (29.8%) had a tubal factor, 31 (25.8%) had ovulation disorders, 6 (5.0%) had uterine factors, and 48 (40%) were normal. The characteristics of male patients examined in this study were that 80 (66.7%) were normal, 13 (10.8%) had oligospermia, 8 (6.7%) had ashenospermia, 2 (1.7%) had teratospermia, 10 (8.3%) had oligoasthenospermia, and 6 (5.0%) had azoospermia.

The mean ages of groups I and II were 38.8; 1.8 and 39.5; 1.8 years, respectively (p=0.17). The number of retrieved oocytes, endometrial thickness, and number and quality of transferred embryos were similar between the 2 groups (Table 1). Twenty clinical pregnancies were confirmed using a urinary pregnancy test and by the presence of gestational sacs on transvaginal ultrasound. In group I, 13 clinical pregnancies (13.6%) were confirmed. Seven of these were aborted, and 8 take-home babies (4 singletons and 2 sets of twins) were recorded. Seven pregnancies were confirmed in group II, with 3 abortions, 1 ectopic pregnancy, 1 set of twins, and another 2 singleton pregnancies. The early pregnancy loss rate included 7 abortions in group I, and 3 abortions and 1 ectopic pregnancy in group II; no difference was seen between the 2 groups (p=0.63, Fischer's exact test).

| Table 1. Comparison of the Influence of LAH on Women Aged over 37 Years |
|-----------------|--------|--------|-----|
| Group I | Group II | p      |
| No. of cases | 80 | 40 |  
| Age (years) | 38.8; 1.8 | 39.5; 1.67 | 0.170 |
| Endometrial thickness (mm) | 12.2; 3.9 | 12.1; 3.0 | 0.850 |
| Oocytes retrieved | 8.7; 6.0 | 8.5; 6.7 | 0.853 |
| Embryos transferred | 3.0; 1.2 | 3.5; 1.5 | 0.710 |
| Embryo score | 2.9; 1.0 | 2.7; 0.9 | 0.280 |
| Implantation rate (%) | 7.3 | 6.7 | 0.648 |
| Pregnancy rate (%) | 16.3 | 17.5 | 0.864 |

*: mean; S.D; tested by Student's t-test.

: Student's t-test.

: χ² test.

We subdivided the women into 2 subgroups: those 37-39 and those older than 40 years. For women younger than 40 years, all parameters mentioned above were compared again. The pregnancy rate seemed to be higher in group II but did not reach statistical significance (Table 2). Women older than 40 years demonstrated similar results (Table 3).
DISCUSSION

Assisted hatching (AH) is based on the hypothesis that alteration of the zona pellucida, either by drilling a hole in it or by thinning it, will promote the hatching of embryos. Microsurgically fertilized embryos with artificial gaps in their zonae appear to have high rates of implantation. AH was first tested by introducing small incisions in the zona of human 4-cell embryos using PZD. The preliminary results were promising. However, PZD was found to have some detrimental effects, such as trapping of embryos, damage during transfer, and lower rates of outgrowth of trophoblasts. The incidence of embryo trapping can be reduced by controlling the size of the holes with a subsequent increased hatching rate, but improvements in the implantation or pregnancy rate are still equivocal. With controlled PZD, an earlier study demonstrated that assisted hatching could improve implantation and pregnancy rates for infertile couples with repeated IVF failures. In the current study, relationships of PZD to female age and zona thickness were not analyzed. Another study demonstrated that assisted hatching with PZD could improve pregnancy rates for women over, but not younger than, 38 years. Another study failed to show any significant benefits from assisted hatching for the factors of advanced female age (≥38 years), zonal thickness, or previous IVF failures.

Acid Tyrode’s solution was introduced to avoid the detrimental effects of PZD. Rates of complete hatching and outgrowth of trophoblasts were higher in embryos artificially hatched with acid Tyrode’s solution. Those embryos unable to escape from the zona were hatched after treatment in acid Tyrode’s solution. Although the use of acid Tyrode’s solution may cause a high rate of cleavage arrest at the morula and blastocyst stages, it has been widely used in recent years. A preliminary study revealed that using acid Tyrode’s solution could enhance implantation in women of advanced age (over 35 years, especially those ≥39 years) with poor prognoses (day 3 FSH > 15 mUI/ml or zona > 15 µm). Implantation and pregnancy rates were improved by AH in women over 39 years. Another study showed enhancement of implantation in women aged between 35 and 42 years. Unfortunately, several later studies failed to demonstrate any benefit in women of advanced ages.

In comparison with acid Tyrode’s solution, diode laser (lambda of 1.48 µm) zona drilling is the latest technology being used for assisted hatching. With this technology, it is not necessary to use a holding pipette or to change the medium while performing hatching. The procedure is safe and efficient. In a mouse model with 1.48-µm diode laser zona drilling, the implantation rate was increased. However, studies on diode laser drilling are rare. One study using women of advanced age (38 years or older) with 2 previous IVF failures showed that total breaching of the zona failed to produce any advantages in implantation and pregnancy or in lowering the rate of miscarriages.

We were unable to find any significant differences in rates of implantation, pregnancies, and early...
pregnancy loss between the groups with and without LAH. Hence, a power analysis was conducted to examine if the non-significance was mainly attributable to type II errors. We found that the power for the analysis of implantation rate (7.3% vs. 6.7%) was less than 0.1. Power analysis also suggested that an extremely large sample, more than 20,000 patients to be exact, would be needed for this difference of 0.6% to be statistically significant. However, we consider that a difference of 0.6% is clinically very small, implying that there was hardly any difference between the 2 groups. A more meaningful interpretation is therefore that LAH is not effective in improving implantation or pregnancy rates in women of advanced age.

Our data failed to demonstrate any benefit of LAH to women of advanced age, and the result may be related to the patterns of zona manipulation or the timing of embryo implantation. AH can help embryos escape from the zona, but it does not change the timing of implantation. The failure of LAH to enhance implantation and pregnancy can be explained by the sudden change in the environment inside the zona after creation of a hole, e.g., loss of embryonic autocrine growth factors, by loss of the protective effects of the zona, and by insufficient variability created by zona drilling as used in this study.

The pattern of zona manipulation may be critical. However, zona thinning or puncture is still being debated. It has been shown that zona thinning alone is insufficient to promote implantation, suggesting that the inner layer of the zona has to be fully breached. A study of mouse embryos revealed that cruciate zona thinning with Tyrode’s solution produced earlier and higher complete hatching rates than did zona hole-drilling controls. Recent studies demonstrated that partial thinning of the outer layer of the zona might be beneficial, either in enhancing the hatching rate of blastocysts, or in improving the implantation and pregnancy rates in women older than 37 years. Characteristics of the inner surface of the zona and the role of the variability of zona thickness remain to be clarified.

Pregnancy outcomes of women of advanced age are influenced by multiple factors, such as oocyte quality, endometrial priming, ovarian reserves, and responses to exogenous gonadotropins. It is obvious that to improve IVF outcomes in women of advanced age, factors other than LAH should be considered.

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雷射人工孵化對於高齡婦女行試管嬰兒之影響：初步報告

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背 景：對於大於37歲的不孕症女性，一般認為胚胎無法突破透明帶而著床和著床率無法提高有關。因此藉由輔助性孵化，應是可以提高著床率。

方 法：本實驗分析120個試管嬰兒治療週期，將實驗對象依年齡分為兩組：第一組為對照組，胚胎在植入之前不做雷射輔助性孵化 (LAH)，而第二組則在植入之前施行LAH。雷射為波長1.48µm (遠紅外線) diode 雷射。雷射打穿透明帶，製造一個約20µm的開口。

結 果：兩組實驗對象的平均年齡分別為38.8 ±1.8及39.5 ±1.6歲 (p = 0.17)。取出之卵子數目、子宮內膜厚度、植入胚胎之數目及分數無明顯統計上之差異。著床率分別為7.3%及6.7% (p = 0.89)，懷孕率分別為16.3%及17.5% (p = 0.86) 以及早期妊娠喪失均無統計上之差異。

結 論：LAH本身對大於37歲的不孕症女性無明顯助益。

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關鍵字：遠紅外線雷射，人工孵化，高齡婦女。