A comparison between quarter, partial and total laser assisted hatching in selected infertility patients

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BACKGROUND: The object of this study was to evaluate the efficacy of laser assisted hatching (LAH) of embryos on implantation and pregnancy rates of a selected group of infertility patients. METHODS: A total of 322 cycles using LAH was undertaken in our Centre between June 1998 and September 1999. Patients were offered LAH if they fell in either one or more of the following categories: (i) Patients over 37 years of age undergoing either IVF or intracytoplasmic sperm injection (ICSI) treatment cycles; (ii) patients with more than 2 previous treatment cycle failures; (iii) patients undergoing frozen embryo replacement cycles and (iv) women who were considered to be poor responders. The initial results of totally breaching the zona pellucida (total LAH; group 1) did not meet with our expectations. We subsequently modified the technique to thinning one area of the zona pellucida (partial LAH; group 2) and this thinned area was then extended to a quarter segment (quarter LAH; group 3). RESULTS: In group 1, the pregnancy rate was 14.6% with a clinical pregnancy rate of 5.2%. In group 2 the pregnancy rate was 20.9% with a clinical pregnancy rate of 18% and for patients in group 3 the pregnancy rate was 29.0% with a clinical pregnancy rate of 22.1%. CONCLUSIONS: Overall there was firm statistical evidence that the pregnancy and clinical pregnancy rates arising from quarter LAH were higher in comparison with partial and total LAH.

Keywords: Assisted hatching/embryos/IVF/implantation/laser assisted hatching

Introduction

The poor implantation rate after the transfer of apparently normal looking embryos is one of the unsolved problems incurred in IVF. Besides intrinsic embryo abnormalities or defective uterine receptivity, hatching failure could also partly explain the low implantation rate in IVF. Blastocyst hatching and implantation may be impaired in some patients where the zona pellucida (ZP) is considered too thick (Cohen et al., 1992), this thickening of the zona being correlated with basal FSH level and preovulatory oestradiol (Loret De Mola et al., 1997). Secondary zona hardening is also thought to occur during in-vitro culture (De Felici and Siracusa, 1982) or after cryopreservation (Carroll et al., 1990). It has been hypothesized that assisted hatching may enhance embryo implantation, not only by mechanically facilitating the hatching process, but by also permitting early embryo endometrium contact.

One of the problems with assisted hatching has been the requirement of extensive technical skill to produce uniform and standardized holes using an acid solution with micro tools, operated under the control of micromanipulators. The drilling of the ZP by laser has been proposed as an alternative to these traditional methods of assisted hatching (Tadir et al., 1991).

Laser and light delivery systems have significant advantages over the chemical or mechanical drilling procedures in that they provide touch-free objective-delivered accessibility of laser light to the target, with minimal absorption by the embryos (Germond et al., 1995). Secondly, the lasers now used for this purpose are affordable and easily adapted to any existing inverted microscope. Furthermore, the laser target in the reaction process is controlled accurately and has been shown to produce the opening in the ZP with no mechanical, thermal or mutagenic side effects (Germond et al., 1995). On this basis, and after the licence for laser assisted hatching was granted to our centre by the Human Fertilisation and Embryology Authority (HFEA), we introduced laser assisted hatching in the UK. Our initial results using the laser to create a single hole completely through the ZP were disappointing. We therefore modified our laser technique to thinning the zona in one particular area. This small change seemed to improve our results significantly. Following this development, and bearing in mind the changes that normally happen in the natural, non-assisted hatching blastocyst process, we speculated that laser application to an extended area of the ZP, rather than at a point, would perhaps further improve our pregnancy rates. Since the site along the zona where natural hatching is likely to take place cannot easily be identified, increasing area of zona thinning was thought to encompass these sites and facilitate this process. In this paper, we present a retrospective analysis of our treatment results comparing the three types of laser assisted hatching (LAH).
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Materials and Methods

Patient and embryo criteria
A total of 322 LAH cycles were performed from the June 15, 1998 until the September 30, 1999 at the London Gynaecology and Fertility Centre, a private clinic in London. LAH was offered to all patients undergoing treatment at our centre that met the following criteria (approved by HFEA): (i) Patients 38 years of age and older; (ii) Patients having frozen embryo replacement; (iii) Two previous IVF or ICSI failed cycles; and (iv) Patients requiring high dose gonadotrophins, i.e. more than 50 ampoules, or a dose of 5 or more ampoules per day.

All patients received written information about laser-assisted hatching and were given an opportunity to discuss this technique with their clinician before signing the relevant consent form.

Total LAH was performed on embryos from 77 patients who fitted in one or more of the above categories. Embryos from 158 patients underwent partial assisted hatching with a further 87 patients embryos undergoing quarter LAH. Patients who agreed to LAH underwent routine IVF/ICSI treatment cycle procedures in order to generate embryos for transfer. The stimulation protocols for IVF and ICSI have already been previously outlined (Meniru and Craft, 1997; Craft et al., 1999). No patients undergoing a frozen embryo replacement were included in this study.

On day 1 of embryo culture, assessment of fertilization was performed and only those embryos exhibiting 2 definite and clear pronuclei were replaced in a fresh culture well of a 4-well multidish (Nunclon, Denmark) containing 0.5 ml of IVF-50 medium (IVF Science, Sweden) overlaid with 0.4 ml of liquid paraffin oil (IVF Science, Sweden). Approximately 1–5 embryos per well were cultured together for a further 24-hours at 37°C in an incubator with an atmosphere of 5% CO₂ in air.

On the morning of day 2, the embryos were scored, and up to 3 embryos deemed suitable for transfer were selected for assisted hatching. It was preferable that the embryos selected for transfer had a minimum cumulative embryo score of 12, i.e. 4 cells with a grading of at least 3 out of 4, or 3 cells with 4 out of 4 grading. The cumulative embryo score was obtained by multiplying the number of cells with the embryo grade (grade 1–4), which was based on the degree of fragmentation. An embryo with a grading of 4 showed no fragmentation, whereas a grade 3 embryo contained up to 25% fragmentation. Any remaining embryos of similar quality were frozen. These supernumerary embryos were frozen intact and not drilled using the laser. Embryos deemed unsuitable for cryopreservation, did not undergo assisted hatching and were discarded. Embryos that were deemed suitable for transfer, and had preferably divided overnight in culture, were eligible for laser hatching. Fourteen days post embryo transfer patients undertook a pregnancy blood test. Only women with a βHCG >25 IU were considered positive. At 7 weeks gestation a
clinical pregnancy was established with the presence of a fetal heart beat.

**Microsurgical laser hatching**

Due to the specific wavelength used, laser drilling was performed directly on the embryos in the 4-well multidish (Nunclon), therefore keeping the embryos in their original culture medium. The dish was placed onto the displacement stage of the diaphot inverted microscope (Nikon, Japan) fitted with a Fertilase micro drill (Fertilase™, Medical Technologies, Montreux SA, Switzerland). The set up used for ZP microdrilling was similar to that described in detail elsewhere (Rink et al., 1994; Germond et al., 1995). Briefly, the laser micro-surgical system consisted of an invisible laser diode beam emitting at a wavelength of 1.48 μm (surgical laser), which was collimated and matched with a 1 mW visible 670 nanometer diode laser aiming beam. These beams were fed into the inverted microscope via the fluorescent port through several mirrors and focused by a 40× microscope objective. The beam focusing through the microscope led to a measured spot size (~8–10 μm) that was magnified and observed on an external monitor. Laser drilling was achieved in either one or two irradiations at a typical power of 47 mW and, for human embryos, an exposure time of 20 ms was required. Changing the irradiation time could control the size of the generated aperture. Under these conditions however, the exposure to the laser light was short enough to ensure precise and localized lysis of the ZP, and therefore it was not necessary to hold each embryo with a suction pipette.

With the help of the displacement stage, a region of the ZP where the perivitelline space was widest was positioned at the location of the aiming spot. The diameter of the ZP covered ~30 to 40% of the screen monitor, thus ensuring the aiming spot could be accurately placed between the middle and outer edge of the ZP. Exposing the ZP to laser light was achieved by using a foot pedal to control the switch. The irradiation time of 20 ms allowed a hole to be drilled at a measured spot size (~8–10 μm) that was magnified and observed on an external monitor. Laser drilling was achieved in either one or two irradiations at a typical power of 47 mW and, for human embryos, an exposure time of 20 ms was required. Changing the irradiation time could control the size of the generated aperture. Under these conditions however, the exposure to the laser light was short enough to ensure precise and localized lysis of the ZP, and therefore it was not necessary to hold each embryo with a suction pipette.

Table I. Pregnancy and clinical pregnancy rates (%) per embryo transfer following transfer of embryos derived from the 3 groups of laser assisted hatching (LAH).

<table>
<thead>
<tr>
<th></th>
<th>Total LAH</th>
<th>Partial LAH</th>
<th>Quarter LAH</th>
</tr>
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<tbody>
<tr>
<td>Number of patients</td>
<td>77</td>
<td>158</td>
<td>87</td>
</tr>
<tr>
<td>Average patient age</td>
<td>37.8</td>
<td>38.9</td>
<td>37.2</td>
</tr>
<tr>
<td>Total number of embryos hatched</td>
<td>213</td>
<td>428</td>
<td>234</td>
</tr>
<tr>
<td>Average number of embryos transferred per patient</td>
<td>2.76</td>
<td>2.70</td>
<td>2.68</td>
</tr>
<tr>
<td>Pregnancy rate per embryo transfer (%) ± SEM</td>
<td>14.6 ± 4.0</td>
<td>20.9 ± 3.2</td>
<td>29.0 ± 4.9</td>
</tr>
<tr>
<td>Implantation Rate (%) ± SEM</td>
<td>2.8 ± 1.6</td>
<td>9.1 ± 2.3</td>
<td>8.1 ± 3.0</td>
</tr>
<tr>
<td>Clinical pregnancy rate per embryo transfer (%) ± SEM</td>
<td>5.2 ± 2.5</td>
<td>18.3 ± 3.0</td>
<td>22.1 ± 4.1</td>
</tr>
</tbody>
</table>

Irradiations were generated until the 3 o’clock position of the embryo was reached.

**Statistical analysis**

The data were analysed by means of logistical regression. The dependent variable was consequently the logistic transfer of the pregnancy rate, and the potential explanatory variables included in the analysis were: the age of the patient, the number of embryos transferred, the three types of laser hatching, and the four categories of patient. Although the analysis was conducted on the logistic scale, the summary tables displayed below consisted of proportions, having been derived by back transformation of the data.

### Results

A summary of the results comparing the three types of hatching is presented in Table I. There were 11 pregnancies (14.6% per transfer) in the total LAH group as compared with 33 (20.9%) in the partial LAH and 25 (29.0%) in the quarter LAH groups. Implantation rates followed a similar trend with 2.8% in the total LAH group, 9.1% in the partial LAH and 8.1% in the quarter LAH group. The clinical pregnancy rate was 5.2% (4/77) versus 18.3% (29/158) and 22.1% (19/87) in the respective groups. These values were corrected for all factors (categories of patients and number of embryos transferred) included in the analysis. Overall, there was good statistical evidence that both partial and quarter LAH were effective in improving the pregnancy rate when compared with total LAH ($P < 0.001$).

The early miscarriage rate (Table II) appeared to be greater for those patients whose embryos had undergone total LAH (49.2%). In comparison, the miscarriage rates for both partial and quarter LAH were similar (17.6 and 19.5% respectively), almost one third that of the total LAH group. The multiple pregnancy rates were also examined (Table II). There were no multiple pregnancies in the total LAH group, however, there were 4 twins, 2 triplets, and one quadruplet pregnancy in the partial LAH group, giving a multiple pregnancy rate of 23.3%.
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There were also 3 twin and 2 triplet pregnancies in the quarter LAH group, giving a 27.7% multiple pregnancy rate.

The number of embryos transferred displayed the customary positive correlation with increased pregnancy rate (Table III). A statistical comparison of this was hampered as most of the patients were in the three embryo replacement group. There was however no evidence of a systematic effect due to patient category. The figures in Table I demonstrated an effect of hatching on the pregnancy rate, which became more emphatic when the clinical pregnancy rate was considered.

Discussion

LAH was offered to patients that were selected on criteria based upon their age (>37 years), previous failures with fresh and frozen embryo treatment cycles or if they were considered poor responders and required high doses of gonadotrophins, for follicle stimulation. Overall, the types of patients with the various indications for LAH were similar in all 3 groups receiving the different types of LAH. Patients whose embryos were subjected to total LAH also received steroids and antibiotics following their embryo transfers in anticipation of hatching. The steroids were given to suppress any possible immunological destruction of the embryos through the breach in the ZP whilst the antibiotics were given to prevent infection. Some clinics utilize steroids and antibiotics on a routine basis for all embryo transfer procedures, regardless of whether assisted hatching is performed or not and these drugs on their own have not been shown to influence the pregnancy rate (Cohen et al., 1990).

Our results strongly suggest that the outcome of LAH is dependent on the mode by which the ZP is breached, as well as the size of the artificial gap. Total LAH was introduced first in our centre based on the literature and information presented on LAH at that time (Germond et al., 1995, 1996, 1998; Antinori et al., 1996). It has also been shown that zona thinning alone is not sufficient in promoting implantation, suggesting the inner layer of the human ZP has to be fully breached (Tucker et al., 1993). The pregnancy and clinical pregnancy rates in this group of patients did not fulfill our expectations. The pregnancy rate in this group was 14.6% and subsequent clinical pregnancy rate dropped to 5.2%. The possible explanations for this relatively bad outcome may have been due to: (i) the breaching of the inner membrane and rapid exposure of the embryos to cells of the immune system. Although patients in this group were administered a low dose of an immunosuppressant to reduce the presence of white cells and any local inflammatory response that might have occurred at the time of embryo transfer, embryos were hatched on day 2 of development thus being exposed to the uterine surface for a longer period of time. Previous studies have suggested that the risk of immunosuppressants at a low dose is not harmful to the embryo (Cohen et al., 1990), (ii) it may be that total laser assisted hatched embryos were at a higher risk of losing blastomeres through the hole as the hatching was performed prior to compaction. (iii) Alternatively, it may be those total LAH drilled embryos were at greater risk of becoming trapped. Published results (Cohen and Feldberg, 1991) showed small holes resulting from application of mechanical zona dissection, inhibited completion of the hatching process rather than improving implantation rates; (iv) using the laser as opposed to traditional chemical or mechanical means of assisted hatching may well be altering the ZP in a different way such that the effect of breaching the inner layer of the zona is detrimental to continued normal development and hatching of the embryo; (v) sudden change in biochemical environment without time for adaptive changes and (vi) finally, detrimental effects of the laser itself although as has been previously suggested (Germond et al., 1995) that the laser had no mechanical thermal or mutagenic effect on the embryos, in view of our results this may not be the case for total LAH.

To try and negate some of these possibilities for the poor outcome of the total LAH, partial LAH was subsequently performed which involved thinning the ZP and not breaching its inner membrane. No steroids or antibiotics were given to this group of patients as the zona was effectively weakened but remained intact. Furthermore, there was no direct hole for blastomeres to potentially escape from or become entrapped. A significant improvement in pregnancy rate (20.9%; \( P < 0.001 \)) and clinical pregnancy rate (18.3%; \( P < 0.001 \)) was obtained in comparison with the total LAH group. Quarter LAH was thought to mimic the physiological process of hatching by creating a greater area of weakened ZP. A significant increase in pregnancy rate (29.0%; \( P < 0.05 \)) and clinical pregnancy rate of (22.1%; \( P < 0.05 \)) was also achieved when compared with the total LAH group.

Although an earlier report suggested a total breach of the human zona was important for successful hatching (Tucker et al., 1993), it may be that the laser has a different effect on the zona in comparison with chemical methods for hatching, particularly if carried out on a human embryo on day 2 of development. Our results suggest that the breaching of the inner zona layer using the laser is detrimental to the implantation potential of the human embryo. Previous data (Khalifa, 1992) observed a beneficial effect on mouse embryo hatching

<table>
<thead>
<tr>
<th>Number of embryos transferred</th>
<th>Number of patients</th>
<th>Pregnancy rate per embryo transfer (%) (± SEM)</th>
<th>Clinical pregnancy rate per embryo transfer (%) (± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>11.2 (6.1)</td>
<td>3.4 (3.2)</td>
</tr>
<tr>
<td>2</td>
<td>38</td>
<td>12.4 (5.7)</td>
<td>13.5 (6.1)</td>
</tr>
<tr>
<td>3</td>
<td>259</td>
<td>24.0 (3.6)</td>
<td>17.9 (2.4)</td>
</tr>
</tbody>
</table>
when a wide area of the outside layer of the ZP was removed, without piercing the glycoprotein matrix completely. It may be that expanding the number of breaking points over a larger area of the ZP is more likely to coincide with the site along the zona where natural hatching is likely to take place.

The differences in miscarriage rates amongst the three groups did not carry statistical significance. However, the large proportion of patients in the total LAH group, who miscarried prior to their ultrasound scans performed at 7 weeks, was of great concern. This seemed to indicate problems with either an incomplete implantation process or poor embryonic development as a result of the total LAH. Whether this was directly due to the total breach of the ZP or other factors associated with the laser is not certain.

The incidence of multiple pregnancies was also considered to be higher than expected, considering the type of patients that were offered this treatment. Whilst the sample size of patients was small, a multiple pregnancy rate of 23.3% in the partial LAH and 27.7% in the quarter LAH group also raised concerns. A similar observation has been made by other authors (Check et al., 1996) suggesting that the occurrence of monozygotic twins may be higher in patients undergoing LAH.

There is considerable variation in the results achieved with assisted hatching between different groups, almost certainly due to the differences in the technical procedures used and also influenced by differences in patient selection for the procedure. With the use of an acid solution, it is difficult to achieve the same degree of uniformity amongst different operators and various situations, and the chemical needs to be washed out to prevent any damage to the embryo. The diode laser offers many advantages over traditional methods in a clinical situation where the ZP needs to be opened. It is also evident that the further studies are needed to fully appreciate the effects of the laser on human embryo development and assess which patients might benefit from assisted hatching. We would like to address this issue with further clinical studies, particularly to assess the effect of LAH in more favourable groups having their first IVF/ICSI. Current protocols are being established to undertake a randomized prospective study comparing women with similar fertility profiles and the significance of partial/quarter LAH.

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