Laser-assisted hatching increases pregnancy and implantation rates in cryopreserved embryos that were allowed to cleave in vitro after thawing: a prospective randomized study

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BACKGROUND: Cryopreservation of embryos may lead to zona hardening that may compromise in vivo hatching and implantation following thawing and transfer. Assisted hatching (AH) has been advocated as a means of assisting the natural hatching process and enhancing implantation. METHODS: The aim of this study was to assess in a prospective randomized manner the effect of laser-assisted hatching (LAH) on implantation as well as clinical and multiple pregnancy rates (the primary outcome) after the transfer of frozen–thawed embryos. All embryos were thawed the day before transfer, and LAH was performed the next day on embryos that cleaved. Control group consisted of embryos that were transferred without AH. RESULTS: The performance of LAH significantly increased implantation (9.9 versus 20.1%, \( P < 0.01 \)), clinical pregnancy (27.3 versus 40.9, \( P < 0.05 \)) and multiple pregnancy rates (16 versus 40.3%, \( P < 0.07 \)). In the LAH group, significantly more excess embryos that were left in culture hatched in vitro. CONCLUSIONS: LAH improves the outcome of frozen–thawed embryo transfer when performed before transfer on embryos that were allowed to cleave.

Key words: cryopreservation/frozen–thawed embryo transfer/implantation/laser assisted hatching/multiple pregnancy/pregnancy rate

Introduction

Assisted reproduction generally results in surplus embryos that can be cryopreserved for later use. The success of a cryopreservation programme will undoubtedly increase the cumulative conception rates attained in IVF/ICSI. Success associated with the transfer of frozen–thawed human embryos has been generally lower than that obtained with fresh embryo transfer. There may be various reasons for this, including embryo preselection, freezing damage and zona hardening. Elasticity and thinning of the zona pellucida are essential for the embryo-hatching process, both of which can be adversely influenced by advancing maternal age, in vitro culture conditions and the freezing–thawing process itself (De Felici and Siracusa, 1982; Carroll et al., 1990; Cohen et al., 1990; Cohen, 1991; Tucker et al., 1991). Assisted hatching (AH) that is based on the presumption of creating artificial openings (slits or holes) in the zona pellucida might assist the in vivo hatching process of embryos. This technique has been shown to increase implantation and pregnancy rates in women of advanced age, in women with recurrent implantation failure and following the transfer of frozen–thawed embryos (Sallam et al., 2003; Gabrielsen et al., 2004).

The aim of this study is to assess in a randomized manner the outcome of frozen–thawed embryo transfers with laser-assisted hatching (LAH) or without LAH (no LAH).

Materials and methods

Patient selection, controlled ovarian stimulation, oocyte recovery and embryo transfer

Three hundred and sixty-six (183 with LAH and 183 with no LAH) thaw–transfer cycles undertaken within a 12-month period were analysed (Figure 1). All couples had been subjected to ICSI for male factor or unexplained infertility in the 24 months before the initiation of the study. During this period, 2312 fresh ICSI/embryo-transfer cycles were performed, of which 789 (34.1%) resulted in the cryopreservation of excess embryos. IVF cases were excluded. The reader is referred to our previous publications for stimulation protocols, oocyte recovery and embryo-transfer techniques (Urman et al., 2003; Balaban and Urman, 2005).

Fresh embryo culture

GIII series sequential culture system (Vitrolife, Gothenburg, Sweden) was used for in vitro culture of fresh embryos. Embryo culture was performed as described previously (Balaban and Urman, 2005).

Endometrial preparation and transfer of frozen–thawed embryos

The women received increasing doses (2 mg for four days, 4 mg for 4 days and 6 mg thereafter) of estradiol (E₂) valerate tablets (Cyclo-Progynova, Schering AG, Istanbul, Turkey) following down-regulation with a GnRH agonist. When the endometrium reached or exceeded 8 mm and a triple-line echo (Lucrin, Abbott, Issy, France) (three-line

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Laser-assisted hatching in frozen–thawed embryo transfer

The appearance of the endometrium as shown in the sagittal section of transvaginal ultrasound was evident, vaginal progesterone (Crinone vaginal gel, Serono, Bedfordshire, UK) was started once a day, and the patient was asked to come for embryo transfer in the morning of the fourth day. The embryos were thawed on day 3 and were kept in culture overnight. Embryos that cleaved or reached the morula stage on the fourth day were selected for transfer.

Freezing protocol
A modified (addition of sucrose) method of Testart et al. (1986) was used for early embryo freezing. 1,2-Propanediol was used as a permeating cryoprotectant. Sucrose which is a large molecule that osmotically promotes dehydration during cooling and protects against cell lysis when thawing was used as a non-permeating cryoprotectant. A phosphate-buffered solution was also used so that all the steps may be performed outside the incubator and at ambient temperature. FREEZE KIT-1 (ref: 10012, Vitrolife) was used as recommended by Vitrolife Fertility Systems products throughout the freezing procedure (Vitrolife GIII Series Manual, 2002).

Only good-quality embryos categorized as G1–G2 (Hardarson et al., 2001) with at least five blastomeres on day 3 were cryopreserved because the cryosurvival rate is related to the initial quality of the embryo (Testart et al., 1987; Karlstrom et al., 1997). Embryo freezing was performed as described in Vitrolife GIII Series Manual. Planer KRYO 10 Series III (Planer Products, Sunbury on Thames, UK) was used for cryopreservation. The straws were manually seeded at -7°C with liquid nitrogen (LN₂)-cooled Mortimer High Security CBS straw-seeding forceps (R30724, Rocket Medical Plc, Washington, UK).

Figure 1. Patient flow through the various stages of the trial.
UK). Following seeding, the embryos were further cooled to –30°C and then to –80°C. Straws were removed and plunged into the LN2 storage tank immediately at the end of the freezing programme.

**Thawing protocol**

The straws were thawed one at a time, and all steps were performed at ambient temperature. Thaw-kit 1 (ref: 10013, Vitrolife) was used for the thawing procedure. All solutions were pre-equilibrated to ambient temperature before use. The manual for recommended use of Vitrolife Fertility Systems was followed for the whole procedure. Embryo-thawing procedure was undertaken preferably 24 h before the transfer to examine further development of the embryo.

**Patient randomization and LAH**

Embryo-thawing procedure was performed 1 day before the embryo transfer, and LAH was performed in the morning of the next day before the transfer. LAH was performed only on embryos that survived following thawing. The assessment of cryosurvival was performed as described by Rienzi et al. (2002). Frozen–thawed embryos were considered to have survived if ≥50% of the blastomeres were intact or had at least three viable cells or one cleaving blastomere after thawing.

Patients were randomized into LAH versus no AH using a computer-generated list of random numbers. Informed consent was obtained from all couples. The Institutional Review Board of the American Hospital approved the study.

A 1480-nm diode laser in a computer-controlled non-contact mode was used for laser hatching (IVF Workstation and Zona Laser Treatment System, Hamilton Thorne Instruments, Beverley, MA, USA). The primary outcome was the implantation rate/Secondary outcomes were clinical pregnancy and multiple pregnancy rates. The IVF Workstation that uses a compact diode laser is attached to an Olympus IX–70 (Olympus, Tokyo, Japan) inverted microscope below the objective turret. Quarter laser-assisted hatching (Q-LAH) was applied to each embryo as described previously (Mantoudis et al., 2001). The thinning of the zona pellucida by laser shots was initiated at one point and continued until 25% of the zona pellucida was drilled (e.g. laser drilling was initiated at the 12 o’clock position, and consecutive shots were applied until the 3 o’clock position of the embryo was reached). Embryos selected for treatment by laser were placed in a Falcon culture dish (ref: 3652, Becton Dickinson Labware, NJ, USA) that contained 10 μl of pre-equilibrated (incubated at 37°C) media (containing the buffer MOPS to avoid pH changes during the procedure) (G-MOPS, Vitrolife, Gothenburg, Sweden) supplemented with human serum albumin (HAS) (ref: 10064, Vitrolife) and covered with paraffin oil (OVOIL-100, ref: 10029, Vitrolife). The culture dish was replaced onto the heated (37°C) basal FSH levels, mean number of previous failed ART cycles and indications for ICSI were similar in the two groups (Table I). Likewise, pre- and post-thaw embryo quality (the percentage of grade 1 and 2 embryos and embryos having eight or more blastomeres on day 3), cryosurvival and progression to the morula-stage rates were also similar (Table II). Significantly more excess embryos that were observed in culture following thawing hatched in vitro in the LAH group (P < 0.05). Clinical results of thaw–transfer cycles are summarized in Table III. Despite the

### Table I. Patient characteristics in the laser and no-laser groups

<table>
<thead>
<tr>
<th></th>
<th>LAH (n = 183)</th>
<th>No AH (n = 183)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean female age (years)</td>
<td>32.4 ± 3.3</td>
<td>32.7 ± 3.1</td>
<td>NS</td>
</tr>
<tr>
<td>Duration of infertility (years)</td>
<td>7.1</td>
<td>6.7</td>
<td>NS</td>
</tr>
<tr>
<td>Basal FSH level (mIU/ml)</td>
<td>4.7</td>
<td>4.6</td>
<td>NS</td>
</tr>
<tr>
<td>Mean number of previous failed ART cycles</td>
<td>1.9</td>
<td>1.7</td>
<td>NS</td>
</tr>
<tr>
<td>Causes of infertility</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>132</td>
<td>134</td>
<td></td>
</tr>
<tr>
<td>Unexplained</td>
<td>28</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Tubal</td>
<td>4</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Mixed</td>
<td>19</td>
<td>16</td>
<td></td>
</tr>
</tbody>
</table>

### Table II. Embryo characteristics before freezing and after thawing

<table>
<thead>
<tr>
<th></th>
<th>LAH (n = 183)</th>
<th>No AH (n = 183)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of embryos frozen (mean)</td>
<td>1097 (5.9)</td>
<td>1135 (6.2)</td>
<td>NS</td>
</tr>
<tr>
<td>Number of G1 embryos frozen (%)</td>
<td>275 (25)</td>
<td>264 (23.2)</td>
<td>NS</td>
</tr>
<tr>
<td>Number of G2 embryos frozen (%)</td>
<td>822 (74.9)</td>
<td>871 (76.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Number of 8-cell embryos frozen (%)</td>
<td>287 (26.1)</td>
<td>256 (22.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Number of thawed embryos (mean)</td>
<td>934 (5.1)</td>
<td>941 (5.1)</td>
<td>NS</td>
</tr>
<tr>
<td>Cryosurvival (%)</td>
<td>878 (94)</td>
<td>881 (93.6)</td>
<td>NS</td>
</tr>
<tr>
<td>Number of G1 embryos after thawing (%)</td>
<td>222 (25.2)</td>
<td>212 (24)</td>
<td>NS</td>
</tr>
<tr>
<td>Number of G2 embryos after thawing (%)</td>
<td>484 (55.1)</td>
<td>503 (57)</td>
<td>NS</td>
</tr>
<tr>
<td>Number of G3 + G4 embryos after thawing (%)</td>
<td>172 (19.5)</td>
<td>166 (18.8)</td>
<td>NS</td>
</tr>
<tr>
<td>Number of morulae on day 4 (%)</td>
<td>492 (56)</td>
<td>504 (57.2)</td>
<td>NS</td>
</tr>
<tr>
<td>In vitro hatching among excess embryos that progressed to morulae on day 4 (%)</td>
<td>73/161 (45.3)</td>
<td>42/129 (32.5)</td>
<td>0.027</td>
</tr>
<tr>
<td>In vitro hatching among excess embryos having 10–14 cells on day 4 (%)</td>
<td>59/186 (31.7)</td>
<td>34/167 (20.3)</td>
<td>0.015</td>
</tr>
</tbody>
</table>

LAH, laser-assisted hatching; NS, not significant.

### Results

Of the 789 couples who had their embryos cryopreserved before the initiation of the study, 366 (46.3%) opted for thawed embryo transfer. Of these, 57 had a clinical or a biochemical abortion, and 309 had failed to conceive in their fresh embryo-transfer cycle. These couples were randomized into thawed embryo transfer following LAH versus no AH. Mean female age (at the time of fresh embryo transfer), duration of infertility, basal FSH levels, mean number of previous failed ART cycles and indications for ICSI were similar in the two groups (Table I). Likewise, pre- and post-thaw embryo quality (the percentage of grade 1 and 2 embryos and embryos having eight or more blastomeres on day 3), cryosurvival and progression to the morula-stage rates were also similar (Table II). Significantly more excess embryos that were observed in culture following thawing hatched in vitro in the LAH group (P < 0.05). Clinical results of thaw–transfer cycles are summarized in Table III. Despite the

### Statistical analysis

The analysis of the results was undertaken using Student’s t-test for numerical and chi-square or Fisher exact test for categorical variables. A P value of <0.05 was accepted as significant. Assuming a pregnancy rate of 35% following frozen-thawed embryo transfers, we calculated that in order to show a 50% increase at 0.05 significance level with a power of 90%, 178 patients were needed in each group. Therefore the study sample size has over 90% power to show a difference of 100% and a power of 70% to show a difference of 50% increase in the implantation rate between two groups.
Outcome of thaw–transfer cycles

<table>
<thead>
<tr>
<th>Number of thaw cycles</th>
<th>LAH</th>
<th>No AH</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryos transferred</td>
<td>183</td>
<td>183</td>
<td></td>
</tr>
<tr>
<td>(mean ± SD)</td>
<td>531 (2.9 ± 0.7)</td>
<td>585 (3.1 ± 0.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Implantation/transferred %</td>
<td>107/531 (20.1)</td>
<td>58/585 (9.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Clinical pregnancy/cycle (%)</td>
<td>75/183 (40.9)</td>
<td>50/183 (27.3)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>First-trimester loss/clinical pregnancies (%)</td>
<td>37/775 (9.3)</td>
<td>65/70 (9.2)</td>
<td>NS</td>
</tr>
<tr>
<td>Multiple pregnancies (%)</td>
<td>31 (41.3)</td>
<td>8 (16)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Singleton/twin/triplet</td>
<td>44/30/1</td>
<td>42/8/0</td>
<td></td>
</tr>
</tbody>
</table>

LAH, laser-assisted hatching; NS, not significant.

Discussion

Our data indicate that LAH before the transfer of frozen–thawed embryos increases implantation and pregnancy rates. The patient population is quite homogeneous, as all were subjected to ICSI and embryos from fresh cycles were generated within a relatively short period of time, thus decreasing the likelihood of being subjected to different culture media and laboratory environment. Patient and embryo characteristics in the two groups were also similar, thus precluding any bias that may have influenced our results. Excess embryos were observed in culture, and it was demonstrated that significantly more of those subjected to LAH hatched in vitro. Provided sibling embryos that were transferred behaved similarly, it may be speculated that LAH acts by enhancing the in vivo hatching process, thus facilitating embryo implantation.

Elasticity and thinning of the zona pellucida are essential for the hatching process, both of which can be adversely influenced by advancing maternal age and in vitro culture conditions (Cohen et al., 1992; Schiewe et al., 1995a; Mandelbaum, 1996). In vitro culture of embryos coupled with the stress induced by the freeze–thaw process may further lead to zona hardening in freeze–thawed embryo-transfer cycles (Carroll et al., 1990; Tucker et al., 1991). Zona pellucida is dissolved in lysine, and quantitative or qualitative deficiencies in its secretion could result in hatching impairment (Gordon and Dapunt, 1993). Suboptimal culture conditions may cause such deficiencies. The trophoectoderm of some embryos may not be able to secrete the hatching factor, and lysine production could be influenced by patient’s age (Cohen et al., 1992; Schiewe et al., 1995b).

Fewer than 25% of the expanded blastocysts have been shown to hatch in vitro, presumably secondary to zona hardening (Fehilly et al., 1985). Conversely, embryos with a thin zona and embryos that have been subjected to microsurgical dissection resulting in artificial gaps in their zona were shown to implant more efficiently (Cohen et al., 1990; Cohen, 1991). These observations led to the development of the so-called AH technique that is based on the presumption that creating artificial openings (slits or holes) in the zona pellucida might assist the in vivo hatching process of embryos that are kept in culture.

AH can be performed using different techniques such as partial zona dissection, acid Tyrode’s treatment and the laser. Different techniques appear to yield similar results in terms of implantation and pregnancy rates (Balaban et al., 2002).

Why AH facilitates embryo implantation is not clear. The enhancement of the embryo–endometrium contact and synchronization may be one of the explanations (Liu et al., 1993). The artificial gap produced by hatching may also serve as a channel for the exchange of metabolites and growth factors and from the endometrium (Cohen et al., 1992).

Some centres use AH for poor prognostic patients such as women with advanced age, poor-quality embryos, embryos with thick zona pellucida and previous implantation failures. Others use AH non-selectively in all couples undergoing IVF. In a meta-analysis of randomized studies in the literature, AH improved the pregnancy rate when performed on all patients’ embryos that were to be transferred regardless of patient and embryo characteristics (Sallam et al., 2003). However, results from this meta-analysis should be interpreted with caution, as there was significant heterogeneity between studies and inadequate data regarding miscarriage rates and other negative outcomes (Edi-Osagie et al., 2003).

AH in frozen–thawed cycles has been shown to increase pregnancy rates in retrospective studies (Check et al., 1996; Tao and Tamis, 1997; Cohen et al., 1999). All these studies used chemical zona drilling. In another retrospective study with historic controls, however, AH by partial zona dissection did not appear to benefit women undergoing thawed embryo transfer (Edirisingshe et al., 1999).

Our study appears to be fourth randomized study that analysed the impact of AH before thawed embryo transfer. Gabrielsen in a pseudorandomized study (allocation based on odd–even dates) showed that acidic Tyrode’s solution increased the implantation rate of cryopreserved–thawed embryos (Gabrielsen et al., 2004). The difference in clinical pregnancy rate although increased by AH did not reach statistical significance most likely due to inadequate number of cycles included in the study.

In a more recent randomized study, however, Ng et al. (2005) failed to show any beneficial effect of LAH on implantation and pregnancy rates following the transfer of thawed embryos. Patients of advanced age similarly did not benefit from LAH. The authors only noticed a trend towards increased implantation rates when the zona thickness was >16 μm. The major difference between our study and the study by Ng et al. is that we performed LAH approximately 20 h after thawing and only on embryos that showed evidence of cleavage. Ng et al. also indicated that their results might have differed if embryos were allowed to cleave in vitro.

In a European multicentre prospective randomized study, Primi et al. (2004) were unable to show any benefit of AH in frozen–thawed embryo transfer cycles. Furthermore, AH appeared to be detrimental in the absence of immunosuppressive treatment. None of the patients in our study received immunosuppressive treatment. Primi et al. froze some embryos at the pronuclear stage and some at the cleavage stage. Results were not analysed according to the stage of freezing. There may be several explanations for different results. First, this is
the only study that showed a dramatic decrease in implantation rates to 1.6% in the LAH group. Second, an outdated version of the laser device was used that did not allow the control of the heat absorbed by the zona pellucida. Third, thawing and transfer were performed in the morning of the same day, thus not allowing further embryo cleavage. Finally, laboratory parameters and endometrial preparation protocols amongst the centres were not clearly stated. All of the above shed doubt on the conclusions reached by the study of Primi et al.

In conclusion, our study clearly showed that LAH increased implantation and clinical pregnancy rates in women undergoing thawed embryo transfer. The strengths of this study are that it includes sufficient number of subjects, it is properly randomized and is undertaken in one centre. Participants were homogeneous, as only ICSI cases were included. Only embryos that showed evidence of cleavage were subjected to AH; thus, embryos with less than optimal viability were excluded. A significant increase in multiple pregnancy rate was observed in the LAH-treated group. The major shortcomings are that it is not blinded, that zona pellucida thickness was not measured and that because of a small number of subjects in the advanced age group, subgroup analysis was not possible.

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