Laser-assisted hatching of zona pellucida – is there an effect on implantation and pregnancy rate?
Preliminary observations
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Abstract
The aim of this study was to determine if laser assisted hatching (LAH) of frozen embryos could improve the implantation and pregnancy rate. Procedure for IVF/ICSI (In Vitro Fertilization/Intracytoplasmic Sperm Injection) was performed in accordance with the standard protocol. Embryos at 4-8 cell stage, which did not show the overall features of degeneration (class A, B, C), were frozen at the third day after fertilization. Zona pellucida incision was performed with a Octax laser. Duration of the action of the laser beam ranged from 2.0 to 2.8 ms. Transfer was performed 3-6 hours after the thaw. In the LAH group of patients there was a significant percentage of early miscarriages; the values of positive beta-HCG, and the rate of clinical pregnancies were 26% vs. 15%. In the group of non-LAH patients the above values were respectively 27.5% and 25%. In conclusion, it appears that laser-assisted hatching of zona pellucida may increase the implantation rate and frequency of pregnancies in some cases. Definitive statement concerning this technique requires a number of randomized studies.

Key words: laser, hatching of zona pellucida, IVF

Introduction
The process of incubating the embryo from the zona pellucida (zona hatching – ZH) is one of the most important factors which determinate the implantation. Under natural conditions, the expansion of the embryo together with the produced enzymes, causes the rupture of the zona pellucida. The final effect of this phenomenon is the release of the embryo (hatching). The cells of the embryo in contact with the endometrium of the uterus start the process of implantation. Other factors affecting the success of implantation include: the quality of the embryo [27] and the condition of the endometrium [9].

The process of incubating of the embryo from the zona pellucida (assisted hatching zona – AZH) has been introduced into IVF programs already in the 90s. Indications for application of this method are: the age of the patient > 37 years, high levels of FSH (usually associated with decreased quality of embryos), thick zona pellucida (above 20 μm), and repeated failed attempts of IVF program.

There are several methods for performing the process of assisted embryo hatching from the zona pellucida: mechanical [8], chemical [14] or by laser [1]. The high efficiency of the laser technique is proved by high percentage of pregnancies [3, 6, 16].

Index of all pregnancies undergoing embryo cryopreservation is usually lower than with fresh embryos. One of the reasons might be worse quality of frozen embryos (the best are used for "fresh" transfers), or the process of hardening of the zona pellucida during freezing-thawing procedure [8].

The aim of this study was to compare the implantation and pregnancy rates after the transfer of frozen embryos with laser-assisted hatching of zona pellucida (LAH), and without disruption the zona pellucida (no LAH).

Material and methods
Procedure for IVF/ICSI was performed in accordance with the standard protocol. Oocytes were aspirated 34-36 h after HCG administration and inseminated (ICSI) after consecutive 3-5 h [13]. The next day, 18h later, the accuracy of conceptions was examined, and then the embryos were cultured for further 48 h. In 2 and 3 day after insemination (ICSI) embryos were evaluated according to the staging proposed by Zieba [27]. The best two or three embryos were selected for transfer. The remaining embryos, with the fragmentation of not more than 20%, were frozen in 0.5 ml straws, with a maximum of 5 embryos in one straw.

Embryos at 4-8 cell stage, which do not show the overall features of degeneration (class A, B, C), were frozen in the third day after fertilization, in the freezer Planer (UK). Specialized program for freezing embryos was used, with 1.2 propanediol as cryoprotectant (Vitro-
life, Sweden).

The freezing program was as follows:
• the beginning of freezing -20°C;
• drop of 2°C /min from 20°C to -7°C;
• seeding at -7°C – stop for 10 minutes;
• drop in temperature of 0.3°C /min from -7°C to -30°C;
• very rapid fall in temperature (30°C /min) from -30°C to -150°C.
• quick transfer of straws with frozen embryos from the freezer compartment to the container with liquid nitrogen (-196°C).

At the day of the transfer, the frozen embryos were defrosted for 30 s at room temperature, then in a water bath at 30°C for 40 s. Then crioprotectant was removed and the embryos were placed in culture medium in an incubator (temperature of 37°C, 5% CO₂). After thawing, the embryos were evaluated in aspect of the number and regularity of blastomeres and the degree of fragmentation of the cytoplasm. Embryos with at least 2 blastomeres were qualified for transfer. Approximately 1 h prior to transfer the embryos were reevaluated, and photographic documentation was performed. If it was necessary to apply the procedure of assisted hatching was applied.

Zona pellucida incision was performed with a Octax laser. Duration of the action of the laser beam ranged from 2.0 to 2.8 ms (figures 1 and 2).

Number of transferred embryos ranged from 1 to 4. Transfer was performed 3-6 hours after thaw, using the Cook catheter 5000 (in case of difficulties with entering the uterus, the catheter Cook 5001 was used).

Results
Research was conducted from January 2006 to April 2008. During this period transfer procedure of thaw embryos was performed in 114 couples, 34 of which were eliminated from the study because, during the process of freezing-thawing of embryos, loss of more than half of the blastomeres occurred. The data from 80 couples were qualified to the final analysis. Six of them repeated the procedure of frozen embryo transfer (86 cycles).

Table 1. Comparison of results between groups of patients with and without laser-assisted hatching

<table>
<thead>
<tr>
<th></th>
<th>LAH</th>
<th>non-LAH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cycles</td>
<td>46</td>
<td>40</td>
</tr>
<tr>
<td>Age of patient</td>
<td>30.8</td>
<td>30.1</td>
</tr>
<tr>
<td>No. thawed embryos</td>
<td></td>
<td></td>
</tr>
<tr>
<td>transferred:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>4</td>
<td>27</td>
<td>18</td>
</tr>
<tr>
<td>Total no. transferred</td>
<td>167</td>
<td>131</td>
</tr>
<tr>
<td>Mean no. transferred</td>
<td>3.6</td>
<td>3.3</td>
</tr>
<tr>
<td>No. of positive beta-HCG</td>
<td>12 (26%)</td>
<td>11 (27.5%)</td>
</tr>
<tr>
<td>No. of clinical pregnancies</td>
<td>7 (15%)</td>
<td>10 (25%)</td>
</tr>
</tbody>
</table>

The difference between the groups that underwent a la-
ser incision of the zona pellucida (LAH), and the control group (without LAH) is in the number of cycles (46 vs. 40). The age of the patients (30.7 vs 30.1), and the number of transferred embryos (3.6 vs. 3.3) were comparable (Table 1). Therefore the results, although reflecting a worldwide trend, should be treated with cautiousness. It is worth to note that in the LAH group of patients there was a significant percentage of early miscarriages; the values of positive beta-HCG, and the rate of clinical pregnancies were 26% vs. 15%. In the group of non-LAH patients the above values were respectively 27.5% and 25%.

Discussion

Zona pellucida is the glycoprotein complex surrounding the ovum, and later the embryo of mammals. It plays an important role in sperm-oocyte interaction [22]. Hardening of the zona pellucida after entering the sperm is a natural process, preventing polispermia, protecting the integrity of the embryo during early development and supporting transport through oviduct. As further divisions of the embryo occur, a zona pellucida becomes thinner, preparing for a natural rupture (blasto-cyst stage). Hardening of the zona pellucida may be also a consequence of embryo exposure to artificial conditions created during the IVF process and later during freezing and thawing [15].

In 1990, Cohen et al. [8] as first proposed the LAH as a method helping the embryo to be release from the zona pellucida. However, the routine use of this procedure is still controversial.

The incidence of pregnancies from frozen embryos, then thawed (FET – frozen-thawed embryo transfer) is typically lower compared with the transfer of fresh embryos, even if their quality – the number and size of blastomeres and degree of fragmentation of the cytoplasm – are comparable. So far, the cause of this difference is not fully explained. Certainly one of the reasons may be changes in the zona pellucida of the embryo, resulting in the hardening during the freezing and thawing [8, 26]. The studies of Gabrielsen et al. [12] and Check et al. [7] showed that the implantation rate was significantly higher in patients whose zona pellucida after thaw was chemically incised using acidic Tyrode’s solution. Similar results were obtained by Tao and Tamis [25], in patients over 38 years, with a thick zona pellucida (>17 μm), poor quality of embryos and several failures in IVF programs.

Yet, other study of Edrisinghe et al. [11], shows even a negative effect of zona pellucida incision in patients above the age of 38 than in the control group.

Whereas, Ng et al. [20] did not show a statistically significant difference in the rate of implantation of frozen-thawed embryos between the groups with and without incision of zona pellucida. At the same time, they suggest that in some groups of patients, the AH procedure may prove useful. The observed rate of implantation after the application of laser zona pellucida incision they received in a group of women over 38 years, with a thick zona pellucida (> 16 μm).

Primi et al. [21] obtained much lower rates of implantation (1.6) and the percentage of pregnancies (1.6) in patients undergoing laser incision of the zona pellucida, especially in the group, which was not treated with antibiotics. After the therapy with antibiotics implantation rate increased to 6.0.

Yet, there were no clear reports that the methods of assisted hatching of the zona pellucida is the best and is associated with the highest rate of implantation (Table 2).

Table 2. Implantation rate and the percentage of pregnancies depending on the method of hatching

<table>
<thead>
<tr>
<th>Authors</th>
<th>Method of hatching</th>
<th>Groups</th>
<th>Index of implantation (FHR) %</th>
<th>Index pregnancies (β-HCG) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gabrielsen et al. 2004</td>
<td>Tyrode acid (O)</td>
<td>AH Control</td>
<td>11.4</td>
<td>17.6</td>
</tr>
<tr>
<td>Ng et al. 2005</td>
<td>Laser (T)</td>
<td>AH Control</td>
<td>9.0</td>
<td>12.5</td>
</tr>
<tr>
<td>Primi et al. 2004</td>
<td>Laser (O)</td>
<td>AH Control</td>
<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
<td>Sifer et al. 2006</td>
<td>Pronase (T)</td>
<td>AH Control</td>
<td>9.6</td>
<td>18.0</td>
</tr>
<tr>
<td>Balaban et al. 2006</td>
<td>Laser (T)</td>
<td>AH Control</td>
<td>20.1</td>
<td>40.9</td>
</tr>
<tr>
<td>Our results</td>
<td>Laser (T)</td>
<td>AH Control</td>
<td>15.0</td>
<td>26.0</td>
</tr>
</tbody>
</table>

AH – assisted hatching with zona opening (O) or thinning (T)

Balaban et al. study from 2002 indicated similar rates of implantation for all methods [2]. But, the last paper of Balaban et al. [3], from 2006, shows that the rate of pregnancies and the percentage of implantation increased significantly after application of laser procedure (LAH) in comparison with the control group (without LAH). Research by Balaban et al. [3] was carried out on a large, homogeneous population. In all cases, ICSI was performed, the breeding and transfer of fresh embryos was carried out in a short time, so it can be assumed that the medium used in the breeding and laboratory
environment remained unchanged. The zona pellucida was incised only in embryos, which during the 20 h of thawing started to dive. Ng et al. [20] also pointed out that their results could differ if the procedure was performed on the embryos which divided after thawing.

There is no consensus whether the zona pellucida should be only incised or a full opening should be performed. Most of the authors [1, 16, 24] advocates only partial zona pellucida incision to avoid such possibilities as loss of blastomer, pathogens entering the interior of the embryo or early abortion. Edgar et al. [10] indicate that the lysis of one of the blastomers during the implementation of a (LAH) procedure can lead to the toxic changes of other cells in the embryo. However, the mechanism is unclear.

Preliminary results of this study do not indicate that the laser incision of the zona pellucida had a significant impact on implantation rate and the percentage of pregnancies. However, to the final conclusions should be carried out on the experience of larger and more homogeneous group of patients.

Our observations also showed, that the majority of pregnancies (75%) of cryopreserved embryos were successful in the next cycle after IVF. It is probably associated with improved uterine receptivity in the natural cycle than in the IVF cycle. Hormonal stimulation of the ovaries leads to a greater number of oocytes, but also worsens uterine receptivity. Pelinck et al. [21] indicate the percentage of implantation of 0-33% in natural cycles, and only 7-9% in the hormone-stimulated cycles. High levels of estradiol (E2) accompanying hormonal stimulation (the greater the more oocytes), does not have beneficial impact on the quality of oocytes and embryos [17]. Lower rate of implantation may be related to the sub-optimal endometrial perfusion [4, 18] and abnormal endometrial morphometry [5]. Ng et al. [19] observed that the flow through the endometrium in the same patients, as measured using 3D ultrasound, was significantly lower in stimulated cycles than in natural. Thus it is possible that the uterine endometrium in natural cycles is characterized by a greater receptivity as compared with stimulated cycles. As the hormonal stimulation of ovaries in not performed in the cycles where frozen-thawed procedure is implemented, it is expected that the index of implantation and the percentage of pregnancies should be higher, unless of course, the quality of embryos is comparable.

In conclusion, there is no clear data proving the benefits of incising the zona pellucida of the embryo. There may be several reasons for such divergent results by different authors: heterogeneous and too small groups or subgroups of patients, freezing of embryos at various stages (2 or 3 days of culture), different time elapsed from thawing to transfer and what is connected with it the ability or inability to observe the divisions of the embryo after thawing. However, given the results obtained by other authors, and our preliminary observations, it appears that laser zona pellucida incision may increase the implantation rate and the percentage of pregnancies in some cases, such as a thick zona pellucida (above 17 μm), advanced age of the patient and previous failures in IVF programs. However, a definitive statement whether the zona pellucida incision significantly increase the percentage of pregnancies and which method is the best, still requires a number of randomized studies to diminish the number of factors influencing the conclusions.

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References


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