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Clinical Assisted Reproduction

Zona Thinning with a Noncontact Diode Laser in ICSI Embryos from Women of Advanced Age

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***Purpose*:** The objective of this study was to determine if the zona thinning (ZT) technique improved the rates of implantation and clinical pregnancy for patients aged ≥38 years submitted to an ICSI program.

***Methods*:** A total of 100 patients submitted to ICSI and aged ≥38 years were divided in a prospective and randomized manner into two groups: Group I – patients submitted to ZT ($n = 50$); a laser diode with 1.48 μm wavelength (Fertilaser) was used for the ZT procedure with 1–2 irradiations of 10 ms applied to four different positions on the zona pellucida (ZP) of each embryo to thin 60–90% of the ZP (each point with a 15–20 μm length of ZT). Group II – patients with no ZT ($n = 50$). In both groups, embryo transfer was performed on the second or third day.

***Results*:** The age of Group I patients (39.8 ± 1.3) did not differ ($p = 0.67$) from that of Group II patients (40 ± 1.9). The number of oocytes retrieved at metaphase II from Group I (6.4 ± 4.2) and Group II (6.8 ± 5) was similar ($p = 0.94$). Normal fertilization rates and cleavage rates were similar ($p = 0.78$ and $p = 0.63$, respectively) for Group I ($71.5 \pm 22\%$ and $96.7 \pm 11\%$) and Group II ($73.5 \pm 19.7\%$ and $96 \pm 11\%$, respectively). The number of embryos transferred was similar ($p = 0.53$) for the two groups (Group I = 3.1 ± 1.3; Group II = 2.9 ± 1.1). The thickness of the ZP of Group I embryos (16.9 ± 2.4 μm) did not differ ($p = 0.97$) from that of Group II embryos (16.9 ± 2.3 μm). The rates of embryo implantation and clinical pregnancy per embryo transfer were similar ($p = 0.67$, $p = 0.61$) for Group I (7 and 16%, respectively) and for Group II (8.2 and 22%, respectively).

***Conclusions*:** These results suggest that ZT in the population aged ≥38 years may have no impact on ICSI success rates. However, this conclusion is limited to a situation in which length of the laser ZT was ≤20 μm and the laser was applied to four different positions.

KEY WORDS: Advanced age; assisted hatching; implantation; intracytoplasmic sperm injection; laser; pregnancy; zona thinning.

INTRODUCTION

Upon reaching the blastocyst stage, the mammalian embryo "hatches" from the zona pellucida (ZP) and undergoes rapid growth of the trophoblast cells for invasion into the uterine epithelium. The hatching process is thought to be caused by digestion of the zona by proteases secreted by the uterus or by alterations in uterine pH. Proteases may also be secreted by the trophoblast cells of the embryo which act on the ZP to facilitate hatching (1).

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It has been suggested that exposure of oocytes and embryos to the artificial conditions of in vitro culture may have negative effects on the embryo's ability to undergo normal hatching and may explain the low rates of implantation following in vitro fertilization (IVF) and embryo transfer (2).

Assisted hatching (AH) of cleaved embryos to increase the implantation and pregnancy rate has been reported by several authors (3–6). It has been postulated that failure of implantation in a substantial number of transferred embryos may be due to their inability to escape from their ZP after reaching the blastocyst stage (1). Advanced maternal age, elevated basal follicle stimulating hormone (FSH) concentrations, and abnormal embryo and ZP morphology have also been proposed to decrease an embryo's ability to hatch and implant (4). Each of the methods currently available to create a gap in the ZP, i.e., mechanical partial zona dissection or zona drilling, chemical zona drilling, and the laser technique, have been reported to have a beneficial effect on implantation rate.

While it seems that embryos with holes in the ZP have an increased potential for earlier and more likely hatching, complete perforation of the ZP does involve certain drawbacks. Holes in the ZP may cause loss of blastomeres, or loss of the whole embryo during contractions of the reproductive tract (7). They also deprive the embryo of its coat, which protects against any detrimental factors in the female reproductive tract. It seems that thinning of the ZP acts optimally to promote both early and complete hatching in the mouse (5).

In 1996, Antinori *et al.* (8) reported that zona thinning (ZT) performed with a laser (Er:YAG) produced a significant increase in implantation and pregnancy rates in a population of women with repeated embryo implantation failure and also among those who were being submitted to IVF for the first time.

The objective of the present study was to evaluate in a prospective, randomized fashion the efficiency of the routine use of ZT with a 1.48 μm noncontact diode laser in embryos from patients aged $\geq$38 years who were being submitted to ICSI.

MATERIAL AND METHODS

A total of 100 patients aged $\geq$38 years whose husbands presented alterations of the male factor according to WHO criteria were submitted to ICSI. The patients were divided into two groups prospectively and at random: I–patients submitted to ZT ($n = 50$), and II – patients not submitted to ZT ($n = 50$).

Ovarian stimulation before the ICSI procedure consisted of blockade of the second phase with nafarelin acetate at the dose of 400 μg/day (Synarel, Searle). After 14 days of treatment with the analogue and the establishment of blockade, administration of recombinant FSH (Puregon, Organon) was started at a fixed dose of 150–300 IU for a period of 7 days. On the eight day of ovarian stimulation, we started monitoring follicular development only by vaginal ultrasound and the doses of FSH were adapted to the ovarian response. When at least three follicles measuring $\geq$17 mm in diameter were observed, hCG was administered at the dose of 5000–10000 IU.

Oocytes were collected from the follicles by ultrasound-guided transvaginal puncture 34–36 hr after hCG. After identification in follicular fluid, the oocytes were classified according to maturity. The cumulus–corona complex was removed by exposure to a hyaluronidase type IV solution (H-4272, Sigma) at the concentration of 40 IU/mL. The denuded oocytes were incubated in IVF-50 medium (Scandinavian IVF Science AB, Sweden) until the time for ICSI. A discontinuous gradient of Sperm-Prep-100TM (Scandinavian IVF Science AB, Sweden) was used for separation of spermatozoa from seminal fluid in the 40 and 90% fractions.

ICSI was performed under an inverted Eclipse TE300 microscope equipped with a Hoffman lens system and coupled to automatic micromanipulators and injectors. A 10% polyvinylpyrrolidine solution (PVP-ICSI-100, Scandinavian IVF Science AB, Sweden) diluted in IVF-50 was used to immobilize the spermatozoa. The ICSI procedure was performed according to the technique described by Svalander *et al.* (9).

Assisted hatching was performed with a laser diode (Fertilaser, Medical Technologies Montreux, Lausanne, Switzerland) with 1.48 μm wavelength operating through an objective lens coupled to an Eclipse TE300 inverted microscope (10). At the time for transfer (Day 2 or 3), the embryos were placed in groups of one to four on Nunc plates containing IVF-50 culture medium. Using the stage of the microscope and no micromanipulators, the embryo was then positioned in the optic field and the ZP was focussed in the direction of the laser light. ZP thinning was performed with 1–2 irradiations of 10 ms applied to four different sites on the ZP of each embryo (Fig. 1) to thin 60–90% of the ZP (each point with a length of zona thinning between 15 and 20 μm). ZP thickness was measured with a scale present in the eyepiece of the microscope.

After the hatching procedure, the embryos were transferred to fresh culture medium in order to avoid

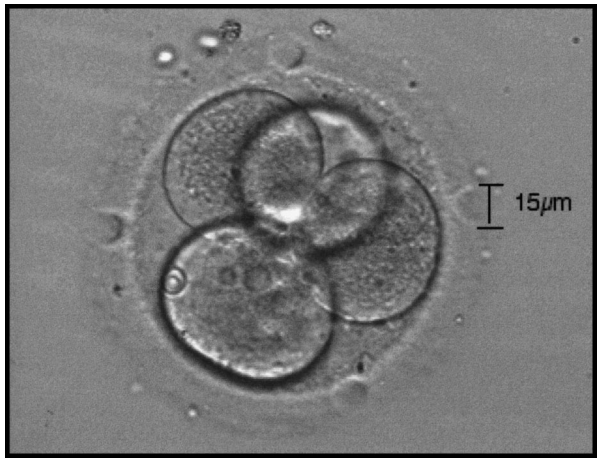

Fig. 1. Embryo with zona thinning applied to four different sites on the ZP.

possible toxicity to the products due to the action of the laser on the organic components of the ZP. Embryo transfer was performed immediately after the procedure (11).

RESULTS

The age of Group I patients (39.8 ± 1.3) did not differ ($p = 0.67$) from that of Group II patients (40 ± 1.9). The number of oocytes retrieved at metaphase II was similar for Group I (6.4 ± 4.2) and Group II (6.8 ± 5.0) ($p = 0.94$). Normal fertilization rates and cleavage rates were similar ($p = 0.78$ and $p = 0.63$, respectively) for Group I (71.5 ± 22% and 96.7 ± 11%) and Group II (73.5 ± 19.7% and 96 ± 11%, respectively).

The number of embryos transferred was similar ($p = 0.53$) for the two groups (Group I = 3.1 ± 1.3; Group II = 2.9 ± 1.1). The ZP thickness of Group I embryos (16.9 ± 2.4 μm) did not differ ($p = 0.97$) from that of Group II embryos (16.9 ± 2.3 μm).

The rates of embryo implantation and clinical pregnancy per embryo transfer were similar ($p = 0.67$, $p = 0.61$; respectively) for group I (7; 16%) and Group II (8.2 and 22%, respectively). The abortion rate was 37.5% for Group I and 45.4% for Group II (Table I).

Table I. Clinical and Laboratory Details of the Groups Studied

	Group I (AH)	Group II (Not AH)	*p*
Patients	50	50	
Age (years)	39.8 ± 1.3	40 ± 1.9	0.67
MII oocytes	6.4 ± 4.2	6.8 ± 5	0.94
Fertilization rate (%)	71.5 ± 22	73.5 ± 19.7	0.78
Cleavage rate (%)	96.7 ± 11	96 ± 11	0.63
ZP (μm)	16.9 ± 2.4	16.9 ± 2.3	0.97
Embryo transferred	156	147	
Mean embryos transferred	3.1 ± 1.3	2.9 ± 1.1	0.53
Number of pregnancies	8	11	
Implantation rate (%)	7	8.2	0.67
Pregnancy rate per transfer (%)	16	22	0.61
Abortion rate (%)	37.5	45.4	
No. of deliveries	5	5	

DISCUSSION

Several days after fertilization the embryo hatches from the surrounding ZP, which is made up of glycoprotein and maintains embryo integrity. Before hatching occurs, the elasticity of the ZP enables the blastocyst to contract and expand, a process which decreases the thickness of the ZP (12).

A common event occurring in embryo culture before implantation is delayed development compared to in vivo conditions (13). This may result in a decreased or blocked cleavage rate, a greater degree of fragmentation, or even alterations in the embryo implantation process. Current research is trying to reduce this effect by formulating new in vitro culture systems or by developing specific methods such as assisted hatching (AH).

The use of AH has been reported to be effective in patients whose embryos suffer chemical or physical ZP changes as a possible consequence of advanced reproductive age, high FSH concentrations, cryopreservation, or unknown causes in populations with repeated implantation failure (14,15). However, there is considerable variation in the results reported by different laboratories after the use of AH, including groups that did not observe any advantage with the use of this technique (16–18). This may be attributed to the type of AH used or to the stages of embryo development during which AH is performed, or yet again to selection criteria and time of embryo transfer.

With respect to age, Schoolcraft *et al.* (19) observed that AH dramatically increases the implantation and pregnancy rates in patients older than 40 years. A retrospective analysis was performed to compare 28 cycles of IVF without AH to 38 cycles of IVF with AH. The delivery rate per oocyte retrieval was significantly higher ($p = 0.0003$) in the AH group (48%) compared to the nonhatched controls (11%). The implantation rate of hatched embryos (22%) was clearly

enhanced compared to the nonhatched embryos (6%, $p < 0.001$).

Recently, Meldrum *et al.* (20) reported that the implantation rates increased with AH in patients aged 35–42 years, with the same incidence of spontaneous abortion or of monozygotic twins.

However, Bider *et al.* (17), after performing AH in a selected group of patients aged >38 years, did not identify an increase in take-baby rate after IVF treatment. A total of 839 embryos from 211 patients aged >38 years underwent AH during 312 cycles of therapy. The outcome of this micromanipulation procedure was compared to 540 nonhatched preembryos transferred to 174 patients during 274 cycles of therapy. AH was performed on four- to eight-cell stage embryos using the zona drilling technique. In the AH group, 839 micromanipulated embryos were transferred (two to four embryos per patient) compared to 540 embryos transferred in the control group. Despite the fact that the pregnancy rate was not statistically different between the groups (8.9% in the AH group versus 5.1% in the controls) a trend toward an increase was noted in the AH group. The implantation rate was 3.75 and 3.55% respectively, and there was no significant difference in abortion rate between the groups. The delivery rate was 3.8 and 3.4% per cycle respectively.

In addition, Lanzendorf *et al.* (21), in a prospective and randomized study, did not observe significant differences in implantation or pregnancy rates after the use of AH in patients aged ≥36 years compared to control. In the cited study, on the day of oocyte aspiration, consenting patients were randomized according to whether all embryos underwent the hatching procedure (hatched; $n = 44$) or all embryos remained unhatched (control; $n = 48$). Patients in both groups were treated with methylprednisolone and doxycycline starting on the day of oocyte retrieval and continuing for 4 days. The hatching procedure was performed approximately 55 h after insemination on all potential embryos for transfer and employed the release of acidified acid Tyrode's medium against the ZP to create an opening approximately 20 μm in diameter. No significant differences were noted in the rates of implantation (11.1% versus 11.3%), clinical pregnancy (39% versus 41.7%), or ongoing pregnancy (29.3% versus 35.4%) between the hatched and control groups, respectively.

On the other hand, it is difficult to define the best length of laser-assisted ZP microdissection. Mantoudis *et al.* (22) observed that clinical pregnancy rates *were higher after* quarter laser assisted hatching (L-AH) in comparison with partial and total L-AH. Blake *et al.* (23) assessed the efficacy and hatching characteristics of in vitro cultured human embryos subjected to laser ZP thinning. ZT was performed on 110 embryos using a noncontact 1.48 μm diode laser and the hatch rate in vitro was compared with 42 control embryos. A maximum of six ablations were made successively around the zona to achieve a depth of 50–80% of the zona thickness for a total length of approximately 80 μm. Scanning electron microscopy was performed on embryos entrapped during hatching to identify the site of hatching. The rate of hatching was significantly higher in blastocysts submitted to laser thinning compared with control embryos (68% versus 33%, $p < 0.01$).

In the present study, a diode laser with 1.48 μm wavelength (Fertilaser) was used for the ZT procedure with 1–2 irradiations of 10 ms applied to four different sites on the ZP of each embryo to thin 60–90% of the ZP. The ZT was performed on four- to eight-cell stage embryos. The rates of embryo implantation and clinical pregnancy per embryo transfer were similar ($p = 0.63$, $p = 0.38$, respectively) for the ZT group (6.3%; 12%) and control group (8.2 and 21%, respectively). However, all embryos included in this study originated from the ICSI procedure and the creation of a small hole in the ZP during the procedure could be the reason why the implantation and clinical pregnancy rates were similar for the two groups.

In conclusion, these results suggest that ZT in the population aged ≥38 years may have no impact on ICSI success rates. However, this conclusion is limited to the situation where the laser ZT was applied to four different sites (each point with a ZT length of 15–20 μm).

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