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Laser-assisted zona pellucida thinning prior to routine ICSI

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BACKGROUND: In MII oocytes showing difficult oolemma breakage, ICSI can cause an increase in the degeneration rate. This may be overcome by laser-assisted ICSI using a 5–10 μm opening in the zona pellucida for injection. However, such a small opening might impair the hatching process, especially if assisted hatching is applied in addition. In order to prevent this, the present study used routine injection through an area of zona pellucida in which laser zona thinning had been applied, providing for both a reduced mechanical stress to the oocyte and assisted hatching. METHODS: This prospective study involved 100 cycles with 1016 MII oocytes. Conventional ICSI (control group) was compared with a modified laser-assisted ICSI (study group) in sibling oocytes. In the latter group oocytes were injected through an extended area of zona thinning. RESULTS: Degeneration rate was significantly lower in the study group ($P < 0.004$). There were no differences in fertilization, or formation and quality of blastocysts. In the study group embryo quality on day 2 was significantly better ($P = 0.004$) and herniation of day 5 blastocysts was increased ($P = 0.005$). Rates of implantation and pregnancy were not affected. However, on day 3 laser-assisted ICSI proved beneficial ($P = 0.038$) in terms of clinical pregnancy rate. CONCLUSIONS: The new method combines a less invasive ICSI technique with assisted hatching. Our preliminary data indicate that in addition to an improved oocyte survival, this new approach increases the hatching rate *in vitro*, which may explain the increase in pregnancy rate, at least in day 3 transfers.

Key words: assisted hatching/blastocyst stage/laser assisted ICSI/oocyte survival/zona pellucida thinning

Introduction

Controlled ovarian hyperstimulation (COH) in subfertile patients allows for a higher number of MII oocytes available for fertilization, although it involves the risk that not all gametes recruited are of the same quality (Imthurn *et al*., 1996; Van Blerkom *et al*., 1997). At least in ICSI, affected oocytes may in part be identified by their morphological alterations (Ebner *et al*., 2001). However, in some patients a suboptimal hormonal supply during COH may cause changes in oocyte quality that are not visible at first glance (Ebner *et al*., 2003a), e.g. both the structure of the zona pellucida (Bertrand *et al*., 1996; Loret de Mola *et al*., 1997) and the elasticity of the oolemma (Amsterdam and Aharoni, 1994; Palermo *et al*., 1996; Ebner *et al*., 2002) may be affected.

The latter can be estimated by different responses of the membrane to the injection pipette during ICSI. In contrast to the very frequent normal response, showing a distinct injection funnel prior to rupture, two rather rare breakage patterns are considered as abnormal (Palermo *et al*., 1996): (i) sudden breakage without any invagination during injection; and (ii) difficult breakage characterized by delayed rupture of the oolemma.

It has been shown that additional manipulation in MII oocytes showing difficult oolemma breakage may cause an increase in degeneration rate (Ebner *et al*., 2001) which, in the worst case (e.g. few oocytes), may result in cancellation of the treatment cycle (Liu et al., 1995). In order to avoid this scenario, a modified injection technique has been suggested (Nagy *et al*., 1995; Ebner *et al*., 2002) combining a pressing and a sucking phase, which appears to retain oocyte survival rate at an adequate level.

Recently, an alternative approach called laser-assisted ICSI (Rienzi *et al*., 2001) has been suggested, and has been successfully applied to patients with diminished oocyte survival in previous cycles (Rienzi *et al*., 2001; Nagy *et al*., 2002). This method involves injection of the oocyte through a laser-created hole in the zona pellucida, which facilitates penetration of all anatomical structures. As a consequence, oocyte survival is increased significantly, as demonstrated in a larger number of cases (Abdelmassih *et al*., 2002).

However, none of the above-mentioned studies took into account a major problem of laser-assisted ICSI, namely the impossibility of localizing the laser-generated hole at later developmental stages (Abdelmassih *et al*., 2002). This

phenomenon is particularly evident at the blastocyst stage, when the embryo expands and the zona pellucida gets thinner prior to the natural hatching process. Thus, if assisted hatching is applied in such embryos, as recommended in embryos derived from oocytes with difficult penetration of the oolemma (Ebner *et al*., 2002), an additional opening is unintentionally created that might impair the hatching process *per se* (Van Langendonckt *et al*., 2000) and/or result in monozygotic twinning (Alikani *et al*., 1994; Schieve *et al*., 2000; da Costa *et al*., 2001).

In order to prevent this possible dilemma, we decided not to perform ICSI through a 5–10 μm hole but through an area of zona pellucida in which laser zona thinning (Blake *et al*., 2001; Mantoudis *et al*., 2001) had been applied. This approach allows for accurate location of the manipulated zona area at later developmental stages and, theoretically, should combine two advantages, namely minimal mechanical stress to the oocyte during ICSI (e.g. increased oocyte survival) and assisted hatching. In order to support this theory, a prospective study was set up comparing this modified form of laser-assisted ICSI with conventional ICSI.

Materials and methods

Over a 3-month period a total of 100 consecutive ICSI cases were involved in this prospective study. The mean (± SD) age of all women was 31.6 ± 4.0 years (range 20–39). Oocytes from the same patient were randomly divided into a study group (laser-assisted ICSI) and a control group (conventional ICSI).

All patients were stimulated with a conventional antagonist protocol using recombinant FSH (Puregon®; Organon, Vienna, Austria) from cycle day 2, and a GnRH antagonist (Orgalutran®; Organon) if one 12–13 mm follicle was present during ultrasound scan (on stimulation day 5 or 6).

In all patients, ovulation was induced with 5000 IU HCG (Pregnyl®; Organon), provided that the lead follicle reached a diameter of ~20 mm and serum estradiol appeared adequate. Oocyte retrieval was carried out transvaginally 36 h after ovulation induction. All eggs collected were incubated for at least 2 h in BM1 (NMS Bio-Medical, Praroman, Switzerland; 6.5% CO_2, 37°C), denuded with hyaluronidase (SynVitro Hyadase®; MediCult, Jyllinge, Denmark) and checked for maturity prior to ICSI.

In the oocytes of the control group, routine ICSI was applied according to our previously published guidelines (Ebner *et al*., 2001). Oolemma characteristics were recorded (Palermo *et al*., 1996), and once ICSI proved difficult the injection pipette was changed to minimize the possible influence of bad quality glass tools on the degeneration rate.

In the study group, the zona pellucida was thinned immediately prior to injection using a non-contact 1.48 μm wavelength diode laser (Fertilaser®; MTG, Altdorf, Germany). Thus, the oocyte was fixed by means of a holding pipette and the glycoprotein matrix was leveled down (at the 3 o'clock position) to ~50% of its original thickness by successively applying five to six laser shots around the zona (Figure 1). Each laser beam had a duration of 6 ms, ensuring that a maximum of 70 μm of the zona was covered (Blake *et al*., 2001). Special care was taken not to lyse the innermost layer of the zona pellucida. Since use of a non-contact 1.48 μm wavelength diode laser has become fully accepted in our laboratory and has proved to be a safe and reliable approach, approval of our internal Institutional Review Board was not sought.

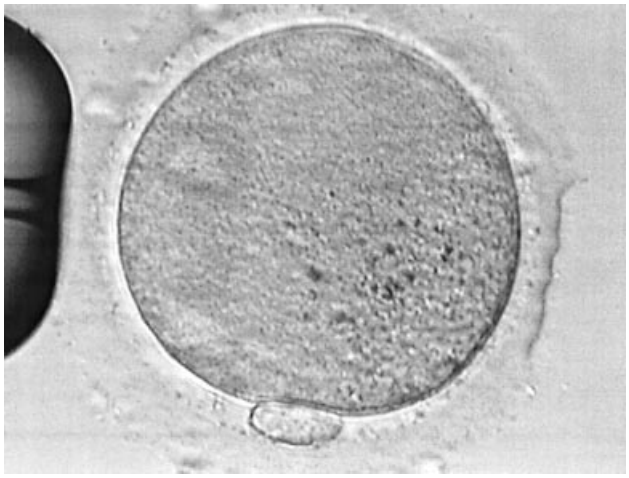

Figure 1. Zona thinning by successively applying five to six laser shots using a non-contact 1.48 μm wavelength diode laser prior to ICSI.

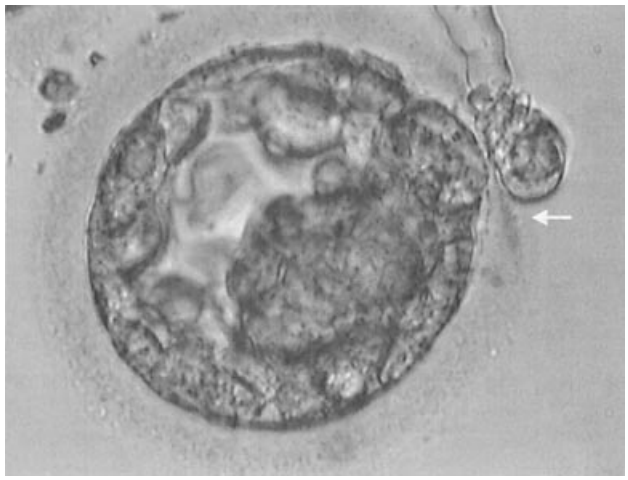

Figure 2. Premature hatching of blastocyst through laser ablation prior to natural zona thinning *in vitro*. Arrow represents 10 μm.

Following ICSI, all oocytes were cultured in groups (20 μl media per oocyte) under sterile filtered, liquid paraffin (MediCult) using BM1 medium (NMS Bio-Medical). On day 1 (16–20 h post-ICSI), oocytes were checked for signs of degeneration and adequate fertilization. Only zygotes clearly showing two pronuclei were further incubated and transferred to Blastassist System Medium 1 (MediCult).

On day 2, cleaved embryos were moved to Blastassist System Medium 2 (MediCult) and a decision was taken regarding the day of transfer. Both number and quality of embryos were considered when allocating couples to day 3 or day 5 transfer. In detail, if at least three embryos with four cells and no or minor fragmentation were available, transfer at the blastocyst stage was considered. If our day 2 prognosis was not supported by day 3 morphology, transfer was brought forward to day 3 ($n = 2$).

In order to avoid any negative influence of embryotoxic ammonium that might concentrate in a small volume of medium, the medium was changed daily up to day 5. Blastocyst expansion and quality were controlled on day 5 according to our modified scoring method (Ebner *et al*., 2003b), originally published by Gardner *et al*. (2000). In addition, the surface of the blastocysts was checked for either the area of zona thinning or any sign of herniation (Figure 2).

Table I. Comparison of preimplantation development *in vitro* between laser-assisted ICSI and conventional ICSI

	Laser-assisted ICSI	Conventional ICSI
Number of MII oocytes	514	502
Fertilization (2PN)	350 (68.1)	332 (66.1)
No fertilization	114 (22.2)	98 (19.5)
1 PN	6 (1.2)	3 (0.6)
3 PN	13 (2.5)	13 (2.6)
Degeneration	31 (6.0)[a]	56 (11.2)[a]
No or minor fragmentation on day 2	237/360 (65.8)[a]	192/348 (55.2)[a]
No or minor fragmentation on day 3	206/360 (57.2)	178/348 (51.2)
Day 4 compaction	142/235 (60.4)	120/229 (52.4)
Day 5 number of blastocysts	111/235 (47.2)	102/229 (44.5)
Good quality blastocysts	34/81 (42.0)	33/82 (40.2)
Hatching blastocysts	27/111 (24.3)[b]	10/102 (9.8)[b]

Numbers in parentheses are percentages. Embryos with minor fragmentation show <10% fragments. Blastocyst quality was evaluated in cases of a clearly distinguishable inner cell mass.
[a]$P = 0.004$; [b]$P = 0.005$.

Morphology of the cleaved embryos or blastocysts was the only criterion for selecting concepti for transfer. Consequently, a certain number of 'mixed' transfers, e.g. one embryo from the study group and one from the control group ($n = 46$), arose. However, the number of homogeneous transfers was found to be adequate for statistical evaluation. Clinical pregnancy was defined as a gestational sac with positive heart activity 4 weeks after embryo transfer.

The χ^2-test was used to analyse nominal variables in the form of frequency tables. Ordinal variables were analysed with the Mann–Whitney *U*-test. Statistical significance was accepted if the *P*-value was <0.05.

Results

Out of a total of 1188 oocytes collected, 1016 were found to have completed meiosis I (85.5%). Regardless of the mode of ICSI, 929 oocytes survived the procedure (91.4%). Degeneration was found to be related to the type of ICSI procedure used, as shown in Table I ($P = 0.004$). An overall fertilization rate of 67.1% (682/1016) was achieved, and no correlation to technique was demonstrated ($P = 0.51$). In addition, no such relationship could be seen in terms of failed fertilization, monopronuclear or tripronuclear zygotes (Table I).

Embryo quality was significantly increased on day 2 using laser-assisted ICSI compared with the control group ($P = 0.004$), since more cleaved embryos in the study group showed no signs of fragmentation at all.

All in all, 213 out of 464 embryos considered for prolonged culture reached the blastocyst stage (45.9%), but blastocyst formation rate was not affected by mode of ICSI ($P = 0.56$). In contrast, with laser-assisted ICSI a significantly increased number of blastocysts could be observed, which already started to bleb out of the zona ($P = 0.005$). Blastocyst quality seemed not to be affected by ICSI technique.

Subdivision of the oocytes according to the response of the oolemma during conventional injection (Table II) revealed that laser-assisted ICSI did not improve survival in oocytes with sudden breakage, but did in oocytes with normal ($P = 0.02$) and difficult ($P = 0.018$) breakage patterns. Further development *in vitro* in both 'abnormal patterns' was not affected by ICSI technique.

A total of 38% (38/100) of patients achieved a clinical pregnancy. The overall implantation rate was 24.0% (48/200). Day 3 (28.6%) and day 5 (47.1%) pregnancy rates did not differ significantly ($P > 0.05$). In addition, implantation rates were comparable between days 3 and 5 ($P < 0.05$).

Table III compares the treatment outcome of laser-assisted ICSI with conventional ICSI. Mode of ICSI does not appear to affect implantation and clinical pregnancy rate. However, if only day 3 transfers were analysed (no decision-making at blastocyst stage), exclusive transfer of study embryos led to significantly better pregnancy rates ($P = 0.024$).

Discussion

There is some evidence that preimplantation development to the blastocyst stage is impaired in ICSI compared with conventional IVF (Shoukir *et al*., 1998; Dumoulin *et al*., 2000; Griffiths *et al*., 2000). In ICSI, there is a theoretical risk of either damaging the spindle apparatus, e.g. in cases where the polar body does not accurately predict the meiotic spindle (Hardarson *et al*., 2000), or of carrying artificial substances such as polyvinylpyrrolidone into the ooplasm, which may affect the further fate of the gametes (Tsai *et al*., 2000). In addition, the cytoskeleton of MII oocytes may be damaged or disorganized by severe mechanical stress during ICSI, as shown in oocytes in which a larger volume of cytoplasm (>6 pl) had been aspirated prior to injection (Dumoulin *et al*., 2001).

A similar situation is found in oocytes that are difficult to penetrate by a sharp injection pipette. In such cases higher pressure must be applied to finally pass all anatomical structures, possibly causing mechanical damage to the meiotic spindle, cytoskeleton or opposite region of the oolemma (Ebner *et al*., 2001).

Faced with a patient who had only oocytes affected in this way, Rienzi *et al*. (2001) developed an elegant method to save oocytes from degeneration. Performing ICSI through a single preformed laser-drilled hole avoids formation of a dominant injection funnel and allows easy entrance to the oocyte without causing leakage of the ooplasm. This method keeps mechanical stress to the spindle apparatus and/or cytoskeleton to a

Table II. ICSI outcome according to oolemma behaviour prior to ICSI

	Normal response		Sudden breakage		Difficult breakage	
	Study	Control	Study	Control	Study	Control
MII oocytes	347	341	24	25	143	136
Fertilization (2PN)	231 (66.6)	235 (68.9)	15 (55.6)	17 (68.0)	104 (72.7)	89 (65.4)
Degeneration	25 (7.2)[a]	42 (12.3)[a]	2 (8.3)	1 (4.0)	4 (2.8)[b]	13 (9.6)[b]
⩽10% fragmentation	159/239 (66.5)[c]	128/238 (53.8)[c]	9/15 (60.0)	9/17 (52.9)	69/106 (65.1)	55/93 (59.1)
Blastocysts (day 5)	73/157 (46.5)	64/159 (40.3)	7/8 (87.5)	6/10 (60.0)	31/70 (44.3)	32/60 (53.3)
Good quality Bc	27/53 (50.9)	22/50 (44.0)	2/7 (28.6)	1/6 (16.7)	5/21 (23.8)	10/26 (38.5)
Hatching Bc	24/73 (32.9)[c]	8/64 (12.5)[c]	0/7	0/6	3/31 (9.7)	2/32 (6.3)

Numbers in parentheses are percentages. Blastocyst quality was evaluated in cases of a clearly distinguishable inner cell mass.
[a]$P = 0.020$; [b]$P = 0.018$; [c]$P = 0.005$.
Bc = blastocysts.

Table III. Implantation behavior and clinical pregnancy rate in homogeneous and mixed embryo and blastocyst transfers

Transfer	Study group only	Mixed	Control group only
Total			
N	33	46	21
Clinical pregnancies	14 (42.4)	19 (41.3)	5 (23.8)
Implantation rate	19/62 (30.7)	20/92 (21.7)	9/46 (19.6)
Day 3			
N	19	18	12
Clinical pregnancies	9 (47.4)[a]	6 (33.3)[a]	1 (8.3)[a]
Implantation rate	9/33 (27.3)	7/40 (17.5)	2/22 (9.1)
Day 5			
N	14	28	9
Clinical pregnancies	5 (35.7)	13 (46.4)	4 (44.4)
Implantation rate	10/29 (34.5)	13/52 (25.0)	7/24 (29.2)

Values in parentheses are percentages.
[a]$P = 0.039$ (study group and mixed group compared with control group).

minimum, a fact that might explain the better embryo quality compared with conventional ICSI (Abdelmassih *et al*., 2002).

To date, laser-assisted ICSI has been generally applied to oocytes showing an increased elasticity of the oolemma (Rienzi *et al*., 2001; Abdelmassih *et al*., 2002) and in oocytes showing an inherent fragility of the membrane (Abdelmassih *et al*., 2002; Nagy *et al*., 2002), or to rescue oocytes after failed fertilization with conventional IVF (Eroglu *et al*., 2002).

The present study is the first to apply a modified version of laser-assisted ICSI as a routine method in a large number of patients. Our data confirm previous findings with regard to increased oocyte survival (Rienzi *et al*., 2001; Abdelmassih *et al*., 2002; Nagy *et al*., 2002). Although the present ICSI approach proved useful in oocytes with normal membrane response as well as in oocytes showing difficult oolemma breakage (Table II), no such benefit could be found in the sudden breakage group. This is in contrast to the literature (Abdelmassih *et al*., 2002; Nagy *et al*., 2002), and may be explained by the fact that serial laser shots on the zona pellucida in the presence of a very fragile membrane may harm the gamete much more than a single one.

A more extensive ablation (~70 μm) circumvents hatching problems possibly arising from the presence of relatively small-sized single laser-drilled hole (Abdelmassih *et al*., 2002). It may in fact be that a small hole in the zona pellucida strangulates the embryo, a phenomenon that may either lead to degeneration of the blastocyst if it cannot escape from the outer shell, or to monozygotic twinning if the emerging embryo is restricted and subsequent splitting occurs. This is supported by disappointing results using a single laser-created hole in order to assist hatching *in vitro* compared with partial zona pellucida thinning (Mantoudis *et al*., 2001).

The dilemma may even get worse if one decides to open the zona pellucida prior to transfer in order to assist hatching, even though the zona has already been lysed to assist ICSI. If the 5–10 μm spot created by a single laser shot cannot be adequately identified, multiple gaps in the zona pellucida will occur and may lead to multiple herniation, known to contribute to monozygotic twinning, at least in the mouse model (Cohen and Feldberg, 1991).

It should be mentioned that Abdelmassih *et al*. (2002) did not observe monozygotic twinning in their study. It is presumed that in this paper all transfers were done using laser-assisted ICSI embryos; the number of clinical pregnancies analysed ($n = 12$) was much too small to allow conclusions to be drawn, since the monozygotic twinning rate in zona manipulated embryos may be estimated to be 1.5–3% (Slotnick and Ortega, 1996), and consequently not even a single monozygotic twin would have been expected. However, our more extensive data indicate that monozygotic twinning is not favoured by the present mode of laser-assisted ICSI.

It should be kept in mind that the percentage of hatching blastocysts on day 5 was found to be decreased in the present study compared with previously published data for zona

thinning (Blake *et al*., 2001). Two things may account for this: first, Blake *et al*. (2001) extended their study up to day 7, which definitely increases the chance of a blastocyst leaving the zona pellucida. Secondly, the authors applied zona thinning at cleavage stage, which allowed thermal ablation of three-quarters of the zona pellucida, whereas we were much more careful (leaving at least half the zona intact), since perivitelline space was rather small at the site of laser application. Therefore, in our study in some cases thinning of the outer shell was perhaps not sufficient to assist hatching.

However, the number of blastocysts that initiated the hatching process *in vitro* was much higher in the study group compared with the control blastocysts deriving from conventional ICSI. It could be demonstrated that every single herniation from the zona pellucida (100%) took place at the site of laser manipulation (Figure 2). This further supports the finding of Blake *et al*. (2001), who did not report any hatching site other than the laser site, which in this study was created on day 2. However, it has to be considered that for methodical reasons the authors could only identify 42.1% (16/38) of the hatching spots positively.

Although it could be demonstrated that our modified laser-assisted ICSI method increases oocyte survival rate, embryo quality on day 2 and hatching rate on day 5, no such correlation could be found with respect to implantation rate and pregnancy rate. This is not surprising, since 38.1% (8/21) of the homogenous transfers out of the control group with conventional ICSI had at least one spontaneously hatching blastocyst transferred as well, a fact that is known to significantly increase implantation rate and pregnancy rate (Balaban *et al*., 2000).

However, if only day 3 transfers were analysed, the clinical pregnancy rate was found to be improved in the study group ($P < 0.05$). Since this benefit could not be observed in day 5 transfers, it seems that morphological evaluation at blastocyst stage provides an additional opportunity to select the right embryos for transfer. It has to be pointed out that in terms of treatment outcome, the present results are preliminary and need to be confirmed by a larger prospective randomized study comparing patients rather than sibling oocytes.

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