Laser assisted immobilization of spermatozoa prior to intracytoplasmic sperm injection in humans

T.Ebner¹, C.Yaman, M.Moser, M.Sommergruber, J.Hartl and G.Tews

Women’s General Hospital, IVF-Unit, A-4020 Linz, Austria

¹To whom correspondence should be addressed. E-mail: Thomas.Ebner@li.lkh.ooe.gv.at

BACKGROUND: The conventional method of immobilization of spermatozoa prior to intracytoplasmic sperm injection (ICSI) is mechanical breakage of the tail by pressing it against the bottom of the injection dish. METHODS: This prospective self-controlled study was set up to evaluate the potential of a non-contact 1.48 µm wavelength diode laser in terms of immobilization. In addition, the fertilization rate and further development potential of such zygotes were investigated. The patients included in our study (n = 60) had oestradiol concentrations >2000 pg/ml, and thus a relatively high number of MII oocytes could be expected. Approximately half the oocytes were injected with laser treated spermatozoa (n = 262, study group) and the other half with mechanically immobilized spermatozoa (n = 252, control group). RESULTS: No significant differences between the two groups in terms of fertilization rate, early cleavage or blastocyst formation were observed. However, time required for identification, aspiration and injection of a potential spermatozoa was significantly shorter in the laser immobilized sperm group (P < 0.001). CONCLUSIONS: The application of a non-contact diode laser for sperm immobilization prior to ICSI is a potentially useful alternative to the conventional mechanical approach.

Key words: blastocyst formation/early cleavage/fertilization rate/immobilization of spermatozoa/non-contact laser

Introduction

Intracytoplasmic sperm injection (ICSI) has been described as beneficial in couples who could not be helped by conventional IVF (Palermo et al., 1992; Van Steirteghem et al., 1993). Though ICSI is more invasive than other micromanipulation techniques (Gordon et al., 1988; Ng et al., 1988; Cohen et al., 1991) this approach has been found to be superior in terms of fertilization and implantation (Palermo et al., 1995).

To keep the fertilization rate constantly high two essential steps in ICSI should be mandatory: (i) rupture of the oolemma and (ii) immobilization of the spermatozoon prior to injection (Catt et al., 1995; Gerris et al., 1995; Van den Bergh et al., 1995; Vanderzwalmen et al., 1996). Since repeated aspiration of the spermatozoa into the injection pipette did not yield adequate results (Van den Bergh et al., 1995) and the use of a piezoelectric manipulation (Huang et al., 1996) did not gain acceptance the only way to carry out immobilization of the gamete has been mechanical breakage of the tail by pressing it to the bottom of the injection dish using a glass tool.

Recently, an alternative method of sperm immobilization has been introduced into assisted reproductive technology (Montag et al., 2000) using a non-contact 1.48 µm wavelength diode laser. When injecting human spermatozoa immobilized using this technique into mouse oocytes, activation and pronuclear formation was seen.

Our prospective self-controlled study was set up to apply this new approach to human oocytes and to evaluate possible differences with the conventional method of sperm immobilization in terms of fertilization, cleavage behaviour, and blastocyst formation. Since day of embryo transfer varied from day 3 to day 6 comparison of pregnancy rates was not possible. Therefore, our decision was to cryopreserve embryos derived with this new method rather than retransfer them. Frozen stored embryos may serve as an alternative source for embryo transfer in all patients involved.

Materials and methods

Institutional Review Board approval was received for this study and prior to treatment, informed consent was obtained from all couples included. In total, 60 patients (mean age 31.3 ± 4.1 years) were evaluated in this prospective study. All women showed oestradiol blood concentrations >2000 pg/ml on the day of ovulation induction (mean ± SEM: 2757.7 ± 674.5 pg/ml) which gave good prognosis to retrieve ≥10 MII oocytes. This number seemed adequate to ensure embryo transfer of ‘conventional’ embryos, since we did not consider embryos derived from laser immobilized spermatozoa for replacement. It was planned to inject approximately half the oocytes with mechanically immobilized spermatozoa (use of a glass pipette; control group) and the other half with laser immobilized ones (study group). Thus, our prospective evaluation was self-controlled. Prior to removal of the cumulus complex oocytes were allocated to the two groups. Sometimes the number of cumulus complexes or the number of mature oocytes differed in both groups. This led to uneven sample numbers (262 in the study group versus 252 in the control group).
Ovarian stimulation was performed according to a long protocol (Smits et al., 1987). In detail, pituitary desensitization was achieved by application of a gonadotrophin-releasing hormone (GnRH) agonist (Suprecur®; Hoechst, Frankfurt, Germany) and stimulation of the ovaries was initiated with an individually adjusted dose of human menopausal gonadotrophin (HMG) (Menogon®; Ferring, Kiel, Germany). Prior to ultrasound guided oocyte retrieval 5000–10000 IU of human chorionic gonadotrophin (HCG) (Pregnyl®; Organon, Oss, The Netherlands) were administered to induce ovulation.

After a 3 h incubation period in 6% CO₂ at 37°C, cumulus cells were removed and added to 80 mU/ml hyaluronidase (MediCult, Hamburg, Germany). Immobilization was carried out at a magnification of ×200 using a high magnification, Olympus, Vienna, Austria) with Hoffman modulation contrast (Modulation Optics Inc., Greenvale, NY, USA) using hydraulic micromanipulators (Luigs and Neumann, Ratingen, Germany).

Spermatozoa of the control group were oriented along the vertical axis using a glass pipette with an inner diameter of ~4 μm (Eppendorf, Hamburg, Germany). Laser immobilization was carried out at a magnification of ×400 by touching the tail of the spermatozoon at least twice. In the study group, spermatozoa were immobilized with a non-contact 1.48 μm wavelength diode laser (Fertilase®; MTM, Montreux, Switzerland) using a double shot technique described elsewhere (Rink et al., 1994; Montag et al., 2000). Briefly, two successive laser irradiations were applied per spermatozoon, the first aimed near the middle of the tail (15 ms; 1.5 mJ) and the second directly on the end of the tail (10 ms; 1.0 mJ). This double shot strategy minimized the total energy dose spermatozoa were exposed to. In addition, laser shots were placed far from the head which made laser application for immobilization a presumably safe process, since we know from scanning electron micrographs of zona pellucida that the highly local damage zone is <1 μm (Germond et al., 1995).

Approximately 18 h after ICSI the presence and number of distinct pronuclei were evaluated. Inspection was repeated 6 h later for signs of pronuclear membrane breakdown and early cleavage. On day 2 (<42 h post-injection), embryo morphology was recorded (number of blastomeres and degree of fragmentation). If the number of good quality embryos on day 3 seemed to be sufficient (Racowsky et al., 2000) further incubation to the blastocyst stage was considered in the control group (blastocyst media, Cook, Sydney, Australia). All cleaved embryos that developed after injection of laser immobilized spermatozoa were intended for prolonged culture to day 5 or 6 and subsequent cryopreservation (CL5000; CryoLogic, Victoria, Australia).

Data were compared using Mann–Whitney U-test, χ²-test, and t-test. Significance was accepted if P ≤ 0.05. Statistical analysis was performed using SPSS software (SPSS Inc., Chicago, IL, USA).

**Results**

Since one of the inclusion criteria for this study was an oestradiol concentration of >2000 pg/ml prior to ovaulation induction, 514 MII oocytes were obtained from 60 patients, with the mean (±SEM) number of oocytes collected being 8.6 ± 2.6. Laser immobilized spermatozoa were used for ICSI in 262 oocytes (51.0%), whereas the conventional technique was used in 252 (49.0%).

For identification of a potential spermatozoon for ICSI, aspiration within the glass pipette and successful injection, the average time needed was 28.2 ± 14.3 s in the control group and 12.1 ± 3.6 s in the study group (P < 0.001). The total rate of zygotes showing two pronuclei (2 PN) was 69.7%, which led to the creation of 358 embryos on day 2. Degeneration after ICSI was detected in 34 oocytes (6.6%).

---

**Table I.** Comparison of treatment outcomes between non-contact 1.48 μm laser immobilization and mechanical sperm immobilization 18–20 h after intracytoplasmic sperm injection (ICSI). Values in parentheses are percentage of total MII oocytes

<table>
<thead>
<tr>
<th>Mode of immobilization</th>
<th>Glass pipette</th>
<th>Non-contact laser</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of MII oocytes</td>
<td>252</td>
<td>262</td>
<td></td>
</tr>
<tr>
<td>Fertilization rate (2 PN)</td>
<td>184 (73.0)</td>
<td>174 (66.4)</td>
<td>NS</td>
</tr>
<tr>
<td>Unfertilized oocytes</td>
<td>40 (15.9)</td>
<td>52 (19.8)</td>
<td>NS</td>
</tr>
<tr>
<td>1 PN or 3 PN</td>
<td>12 (4.8)</td>
<td>18 (6.9)</td>
<td>NS</td>
</tr>
<tr>
<td>Degeneration</td>
<td>16 (6.3)</td>
<td>18 (6.9)</td>
<td>NS</td>
</tr>
</tbody>
</table>

---

**Table II.** Early cleavage behaviour (24 h after ICSI), embryo quality (42 h after ICSI), and developing potential of oocytes obtained from spermatozoa immobilized using two different techniques. Good quality embryos were assessed as the proportion of embryos with <10% fragmentation. Volumes are mean ± SEM

<table>
<thead>
<tr>
<th>Mode of immobilization</th>
<th>Glass pipette</th>
<th>Non-contact laser</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of zygotes (18 h)</td>
<td>184</td>
<td>174</td>
<td></td>
</tr>
<tr>
<td>Early cleavage behaviour (24 h)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>two distinct pronuclei (%)</td>
<td>112 (60.9)</td>
<td>116 (66.7)</td>
<td>NS</td>
</tr>
<tr>
<td>pronuclear membrane breakdown (%)</td>
<td>42 (22.8)</td>
<td>36 (20.7)</td>
<td>NS</td>
</tr>
<tr>
<td>early cleavage (%)</td>
<td>30 (16.3)</td>
<td>22 (12.6)</td>
<td>NS</td>
</tr>
<tr>
<td>Embryo morphology (day 2) mean number of blastomeres</td>
<td>3.7 ± 1.0</td>
<td>3.5 ± 1.1</td>
<td>NS</td>
</tr>
<tr>
<td>mean fragmentation (%)</td>
<td>14.4 ± 10.5</td>
<td>14.9 ± 10.8</td>
<td>NS</td>
</tr>
<tr>
<td>Good quality embryos (%)</td>
<td>64 (34.8)</td>
<td>58 (33.3)</td>
<td>NS</td>
</tr>
<tr>
<td>Blastocyst formation prolonged cultivation</td>
<td>120</td>
<td>174</td>
<td></td>
</tr>
<tr>
<td>blastocyst on day 5 (%)</td>
<td>46 (38.3)</td>
<td>62 (35.6)</td>
<td>NS</td>
</tr>
<tr>
<td>blastocyst on day 6 (%)</td>
<td>22 (18.3)</td>
<td>38 (21.8)</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS = not significant.
No significant differences between the study and control group in terms of fertilization rate or number of degenerated oocytes could be observed (Table I). On day 1 (22–24 h post-insemination) 228 zygotes still showed 2 PN (63.7%). Pronuclear membrane breakdown could be observed in 78 oocytes (21.8%), early cleavage in 52 oocytes (14.5%). Embryo quality (day 2) and the rate of blastocyst formation on day 5 or 6 were not affected by sperm treatment prior to injection (Table II). A total of 168 embryos reached the blastocyst stage on either day 5 (36.7%) or 6 (20.4%).

Discussion

Various lasers have been shown to be valuable tools in a wide field of molecular biology as well as medical research. Their use is appreciated for their precise andatraumatic mode of action. All laser systems have to meet certain requirements, such as minimal thermal effect on the tissue and use of a wavelength sufficiently distant from the maximum absorption of DNA.

In assisted reproductive technology, lasers are mainly used for zona pelluccida drilling to assist hatching. The introduction and establishment of an erbium-YAG laser in IVF laboratories (Feichtinger et al., 1992) brought good results in terms of hatching; however, direct contact to the gamete was required. Therefore, some non-contact types of laser have been developed, making laboratory work much safer and easier (Tadir et al., 1991; Antinori et al., 1996). For some laser systems working in the UV range special optical equipment (Schütze et al., 1994; Antinori et al., 1996) was considered necessary. Other laser equipment made it necessary to change culture dishes or micromanipulators (Feichtinger et al., 1992), therefore the handling of such lasers was quite complicated.

Recently, a non-contact 1.48 μm wavelength diode laser was used in mouse (Rink et al., 1994; Germond et al., 1996) and human oocytes (Germond et al., 1995). In this type of laser, the mode of drilling the zona pelluccida is completely different from other systems since the glycoprotein matrix of the zona pelluccida is disrupted due to thermal effects (Rink et al., 1996). Scanning electron micrographs showed that the local damage zone is <1 μm (Germond et al., 1995).

The ease and precision of microdissection led to alternative applications of the 1.48 μm wavelength diode laser in polar body biopsy (Montag et al., 1998) as well as in the pretreatment of single human spermatozoa prior to cryopreservation (Montag et al., 1999).

The minimal damage zone (Germond et al., 1995) together with the high absorption characteristics of the 1.48 μm wavelength in water (Rink et al., 1994, 1996) makes the non-contact diode laser a safe approach (Montag et al., 2000). In addition, the energy used for sperm immobilization, which is 10 times lower than that used in assisted hatching (Primi et al., 1999), could be significantly reduced using a double shot strategy (Montag et al., 2000).

The finding that human spermatozoa, following immobilization with a laser beam, successfully activated mouse oocytes (Montag et al., 2000) led us to evaluate the fertilization behaviour of laser treated spermatozoa in human oocytes.

Additionally, we focused on the further development potential of such zygotes.

Although even parthenogenetic mechanisms may lead to oocyte activation in humans (De Sutter et al., 1992), the presence of a spermatozoon seems to be indispensable for the further development and implantation potential of embryos. The mechanical stimulus caused by the penetration of the micropipette itself is sufficient to alter the intracellular calcium level (Tesari et al., 1994), but changes in ionic concentrations alone are not sufficient to activate the oocyte completely. A sperm-associated factor is thought to contribute to oocyte activation (Tesari et al., 1994; Dozortsev et al., 1995; Vanderzwalmen et al., 1996). Therefore, to facilitate interaction between such a cytosolic sperm factor and the ooplasm, the membrane of the spermatozoon has to be destabilized to ensure leakage of the sperm factor into the cytoplasm of the oocyte. Alternatively, access may be given to ooplasmic factors directly involved in sperm head decondensation (Van Blerkom et al., 1994).

To date, destabilization of the sperm tail is generally achieved by touching it with the tip of a glass pipette. This procedure can easily be performed by experienced embryologists. Nevertheless, some problems making the immobilization of spermatozoa impossible may be encountered. These phenomena may result from touching the tail with the ICSI pipette at an inappropriate angle (not parallel to the bottom of the injection dish). This three-dimensional problem may be solved by either changing the glass microtool or by repeated sucking of the spermatozoon into the lumen of the pipette. The latter approach was found to be of limited success in terms of fertilization (Van den Bergh et al., 1995), probably because the force applied to the spermatozoon may lead to the destruction of the 9 + 2 system of microtubules leaving the membrane intact. Even if the ICSI procedure was straightforward, it may take up to 1 min successfully to place a spermatozoon into the ooplasm (Palermo et al., 1995). The average time required for injection is strongly influenced by the direction in which the spermatozoon are heading, since the gametes should be oriented along their vertical axis prior to immobilization to minimize the risk of sperm head damage.

Use of a non-contact 1.48 μm wavelength diode laser for immobilization of the spermatozoon (Montag et al., 2000) overcomes these problems because no direct contact to the spermatozoon is required. In addition, this procedure is independent of the spermatozoon orientation within the ICSI dish. Even spermatozoa which do not swim near the bottom of the PVP drop can easily be immobilized. Consequently, manipulation time can be reduced significantly.

As a second advantage of laser assisted immobilization, the magnification of the laser objective (45 times) in combination with the magnification of the monitor allows for better evaluation of sperm morphology according to the WHO (1992). Finally, laser immobilization offers the potential to avoid PVP (Montag et al., 2000), whose use is still controversial (Strehler et al., 1998).

These advantages make the recently described sperm treatment using a non-contact diode laser a potential alternative to conventional handling of the spermatozoa, particularly as no
differences were found in the rates of fertilization, degeneration, cleavage, and blastocyst formation.

Our data confirm the work of Montag et al. (2000) concerning fertilization behaviour of laser treated spermatozoa. In addition, this study is the first to apply successfully laser induced immobilization of the male gamete to a human model. Our study implies that the energy found to be optimal may differ slightly from published data (Montag et al., 2000) without affecting ICSI outcome. This may be due to the fact that all optical systems cause a certain loss of energy and, consequently, usage of different microscopes may lead to different energy values necessary for optimal immobilization.

In times when patient numbers continue to grow, the introduction of an alternative method for the immobilization of spermatozoa prior to ICSI, making the laboratory work simpler and quicker but without lowering fertilization, cleavage or blastocyst formation rates, may be of considerable benefit.

References
Racowsky, C., Jackson, K.V., Cekleniak, N.A. et al. (2000) The number of eight-cell embryos is a key determinant for selecting day 3 or day 5 transfer. Fertil. Steril., 73, 558–564.

Received on March 19, 2001; accepted on August 30, 2001