Possible applications of a non-contact 1.48 μm wavelength diode laser in assisted reproduction technologies

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Recently, one laser system has been introduced in IVF fulfilling all safety requirements, while achieving a high standard of reproducibility in terms of ablation diameter. This 1.48 μm wavelength indium-gallium-arsenic-phosphorus (InGaAsP) semiconductor laser offers a variety of laser applications to the embryologist. On the one hand, zona pellucida of oocytes or embryos can be manipulated in order to facilitate ICSI or biopsy and assist hatching, and on the other, spermatozoa may be paralysed or immobilized prior to usage. To conclude, the 1.48 μm diode laser provides a promising tool for the microdissection of subcellular targets. The diode laser stands out due to the rapidity, the simplicity and the safety of the procedure which is supported by healthy offspring after laser application.

Key words: assisted hatching/biopsy/diode laser/immobilization of spermatozoa/zona pellucida

Introduction
A variety of lasers have been found to be valuable tools in a wide field of molecular biology as well as medical research. In gynaecology, lasers were first applied to reproductive organs in the early seventies of the last century (Kaplan et al., 1973). Further advances in endoscopic technology stimulated adaptation for reconstructive pelvic surgery (Bruhat et al., 1979). Once laser surgery had moved to a cellular level (Berns et al., 1981) lasers soon gained entrance to the field of assisted reproductive technologies where their use was appreciated for the precise and atraumatic mode of action.

Lasers in IVF
Tadir et al. (1989, 1990) were the first to introduce a solid-state laser, namely a neodymium:yttrium-aluminium-garnet laser (Nd:YAG; wavelength 1064 nm) in IVF in order to manipulate spermatozoa. In a next step the Nd:YAG laser system was applied for zona drilling (Tadir et al., 1991) by combining the Nd:YAG device with a potassium-titanyl-phosphate crystal, thus doubling the frequency of the radiation (532 and 266 nm). The major advantage of this approach was that the laser beam was directed via a conventional inverted microscope and focused onto the viewing plane by the objective lens. Though better control of zonal damage was reported when the beam was orientated tangentially (rather than towards the lower pole), the created ablations were still prone to inconsistency (Neev et al., 1992).

Approximately at the same time Palanker et al. (1991) proposed usage of an argon fluoride gas laser (ArF), which is based on the temporary formation of excited molecules emitting at a wavelength of 193 nm. SEM of ArF-laser drilled mouse oocytes revealed uniform, round holes with sharp edges. The diameter of the photoablation was determined by the tip size of the required air-filled glass pipette. The safety of the non-thermal ArF-laser has been stressed since normal litters were obtained from transfer of hatched embryos (Lauffer et al., 1993).

Another two such excimer lasers working in the UV range were the xenon chloride (XeCl), emitting at 308 nm, and the krypton fluoride (KrF) laser (248 nm). The first one facilitated fertilization in subfertile mice once the zona had been opened (El-Danasouri et al., 1993) and worked well being used as hatching assistance in the same species (Neev et al., 1993). The latter one was also used for successful penetration of the zona pellucida in thawed mouse embryos (Blanchet et al., 1992). However, the wavelength of this KrF-laser was far too close to the absorption peak of DNA (260 nm) which definitely limited its application in a human model.

All excimer lasers as well as the nitrogen laser (337 nm) of Schütze et al. (1994) work within the UV range of the spectrum (10–380 nm) and are not considered for usage in routine IVF because of the possible cytotoxicity and mutagenicity of UV light (Kochevar, 1989). In order to circumvent this threatening problem it was strived for the introduction of lasers with emission in the infrared range (>800 nm). A pulsed 2.9 μm erbium:yttrium-aluminium-garnet laser (Er:YAG) was the first to be clinically used in an IVF programme (Feichtinger et al., 1992; Strohmer and Feichtinger, 1992). Successful births after zona treatment with the Er:YAG laser proved the safety of this...
approach (Antinori et al., 1994). However, the effectiveness of this ‘contact’ laser system appeared somewhat limited since photoablation is constrained by the size and the shape of the fibre, the diffraction of light at the tip and the need to keep close contact to the gamete or embryo.

Due to the different absorption behaviour of its 2.1 μm emission in water the holmium:yttrium-scandium-gallium-garnet laser (Ho:YSGG) runs without need for additional micromanipulating (Neve et al., 1995; Schiewe et al., 1995a); however, embryos had to be moved to quartz slides causing superfluous physiological stress, a fact limiting its applicability.

To summarize, it appears that the vast majority of lasers have at least one major drawback raising concerns about safety and sterility. Some work within hazardous UV range (XeCl, KrF, ArF, N) or a range much too close to the absorption maximum of DNA (266 nm Nd:YAG, KrF) and, therefore, cannot exclude a possible harm to genetic structures. Others guarantee optimal interaction with cellular structures since they work with a wavelength highly absorbed by water, but emission is also absorbed by the microscope optics. In these cases additional pipettes (ArF) or fibres (Er:YAG) have to be used to guide the laser beam to the working area which is a possible source of contamination.

1.48 μm wavelength diode laser

To our experience, there is only one laser system fulfilling all safety requirements (infrared range, non-contact mode), while achieving a high standard of reproducibility in terms of ablation diameter. This indium-gallium-arsenic-phosphorus (InGaAsP) semiconductor laser operates with a 1.48 μm wavelength and was first introduced by Rink et al. (1994a).

The set-up of this laser is rather complex and consists of a special set of three mirrors and three lenses. In more detail, the invisible continuous wave laser beam emitted from the 1.48 μm laser diode (emitting area, 2 × 0.2 μm²) is collimated using a microscope objective corrected at the 1.5 μm wavelength (focal length, 4.4 mm; numerical aperture, 0.65). It is then matched with a visible aiming beam (670 nm) by the first mirror which has to be highly reflective for the 1.48 μm beam and semi-transparent for the accompanying 670 nm radiation. Both collinear laser beams are then coupled to the inverted microscope by the remaining two mirrors and a second lens, thus directing them along the microscope optical axis. The focused laser radiation is finally led through the objective (magnification ×45) of the inverted microscope (spot diameter, 8 μm).

Approximately half (55 mW) of the original laser output (120 mW) is available in the object plane corresponding to a power density of 109 kW/cm². However, a limitation of power to 47 mW is recommended (Rink et al., 1996). Due to the characteristics of infrared radiation the mode of action using the 1.48 μm diode laser is a thermal one. However, this photo-thermolysis is limited to a micron-ranged spot at the target structure, e.g. a well-localized lysis of the proteins contributing to the zona pellucida matrix. Since neither bubble formation nor acoustic transient was reported (Rink et al., 1994b) temperature at the focus of the laser beam was estimated to be from 60 to 80°C (Rink et al., 1996).

The target structure most frequently investigated with the infrared laser is definitely the zona pellucida. Using this system, holes of a given diameter can be reproduced with a variation of less than 1 μm. SEM images demonstrate the high quality of the drilling mode since the shape of the drilled trench resembles a perfect cylinder with a smooth surface and regular incision edges (Germond et al., 1995; Rink et al., 1996).

Within a wide range of irradiance hole diameters increase with the logarithm of the irradiation time (Rink et al., 1994a). However, it has to be noted that several parameters, like temperature of culture medium (Rastegar et al., 1996), optical set-up, type of culture dish (Schmolz et al., 2003) and degree of soiling (personal observation), directly affect the dose of the laser energy required in order to generate holes with a wanted diameter. Once individual adjustment of the laser system in a laboratory has been performed, a variety of laser applications is offered to the embryologist.

Manipulation of zona pellucida

As the follicle develops, the granulosa cells multiply and establish extensive processes towards the oocyte. In the cleft between the two zona pellucida (ZP) forms. Despite considerable speculation about the origin of this acellular glycoprotein layer (15–20 μm) it has now been accepted that all zona proteins are synthesized exclusively by the oocyte in a coordinate manner (Epifano et al., 1995). Experiments, primarily in the mouse, have led to the conclusion that zona pellucida in mammalian oocytes consists of three zona proteins. In detail, filaments are constructed of repeating ZP2–ZP3 units which are cross-linked by ZP1 (Wassarman, 1988), thus contributing to the structural integrity of the zona matrix. Recently, characterization of involved genes demonstrated that there are in fact four zona pel- lucida genes (Hughes and Barratt, 1999) and, consequently, four zona pellucida glycoproteins are expressed in the human (Lefèvre et al., 2004).

However, around fertilization zona pellucida has several functions including species-specific sperm binding (ZP3), induction of acrosome reaction in order to prevent polyspermy (ZP2). After fertilization, the zona plays a role in protecting the integrity of the developing embryo and it also assists its oviductal transport. Developing embryos show a gradual thinning of the zona pellucida during in vitro culture (Chan, 1987). Closely associated with expansion this thinning reaches its maximum prior to rupture, which seems to be mediated by zona lysins (Schiewe et al., 1995b) and/or uterine enzymes (Rosenfeld and Joshi, 1981).

In view of the complex functions of the zona pellucida it is likely that any irregularity in composition or thickness, regardless of whether caused by genetic (Rankin and Dean, 1996; Stanger et al., 2001) or environmental reasons (De Felice and Siracusa, 1982; Cohen et al., 1990; Schiewe et al., 1995b), can impair optimal function.

While thickness of the glycoprotein envelope can be measured easily, hardness of the same can only be estimated, e.g. using its response to the injection needle in ICSI (Ebner et al., 2002a). Additional help in order to get information about the actual degree of hardness of the zona pellucida matrix comes from laser studies on mouse concepti (Montag et al., 2000c). In this series of animal experiments a constant laser pulse (0.6 mJ) was
aimed at the zona pellucida at different stages of preimplantation development. Openings in oocytes were larger (17 ± 0.6 μm) as compared to zygotes (13.8 ± 0.7 μm). A second significant reduction in hole size was observed between morula (13.6 ± 0.7 μm) and blastocyst stage (10.9 ± 1.6 μm). In theory, smaller openings, while using the same energy, may be indicative for in vitro zona hardening. Routinely assessing zona pellucida hardness using the diode laser would allow for identification of certain oocyte pools in which further manipulation is recommended in order to improve injection, hatching and outcome.

**Laser-assisted ICSI**

In general, human metaphase II (MII)-oocytes are relatively resistant to damage caused by the ICSI procedure per se, with more than 94% of mature oocytes surviving injection provided that denuded oocytes are of adequate quality and ICSI is inconspicuous (Ebner et al., 2001a). However, some oocytes show deviations from a presumed optimal ICSI since the elasticity of the outer structures (zona pellucida and oolemma), which would normally allow the formation of a distinct funnel prior to rupture, appears somewhat impaired. This is characterized by either sudden breakage or delayed penetration of zona and membrane and is likely to result in increased damage of the gametes (Nagy et al., 1995; Palermo et al., 1996; Dumoulin et al., 2001; Ebner et al., 2001a).

In order to avoid this scenario Rienzi et al. (2001) introduced a novel method in a patient with a known history of bad quality MII-oocytes in four previous trials. In these attempts only seven oocytes of 36 (19.4%) survived due to severe problems in passing the zona (including excessive deformation of the oocyte shape). In a fifth cycle, ICSI was planned with assistance of a 1.48 μm diode laser in order to reduce the resistance of the zona pellucida. Therefore, a small hole (10 μm in diameter) was drilled through which easy entrance to the oocyte could be gained without the slightest sign of deformation. Indeed, 61.5% (8/13) of the female gametes remained intact, five were fertilized (38.5%) and a triple pregnancy could be achieved. Subsequently, Nagy et al. (2002) applied this laser-assisted ICSI technique to a patient with exclusively fragile oocytes in precedent cycles in which nine of 18 oocytes (50%) degenerated. In a third treatment cycle mature oocytes were split up between conventional ICSI (n = 5) and ICSI with laser assistance (n = 6). This group used 3–5 laser pulses to create a 5–6 μm channel which facilitated injection. Only one of six laser-treated oocytes (16.7%) did not survive compared to 60% (3/5) in the sibling oocytes without manipulation.

As a common feature of both case reports (Rienzi et al., 2001; Nagy et al., 2002) they reported a trend towards better embryo quality in laser-assisted ICSI which is probably a result of minimized damage to the cytoskeleton and/or meiotic spindle. This finding was later supported by a prospective study on larger patient numbers (Abdelmassih et al., 2002). In detail, a total of 32 patients showing a degeneration rate >20% in previous attempts were randomly divided between routine ICSI and laser-assisted ICSI. The latter was performed using multiple pulses of <2 ms duration in order to drill a 5–6 μm opening but leaving the innermost layer of the zona pellucida intact. Both a significantly improved (P < 0.01) embryo developmental rate (76.5 versus 57.5%) and a higher (P < 0.0001) survival rate (99.6 versus 84%) could be achieved. These data have recently also been confirmed for rather fragile oocytes (Rienzi et al., 2004).

However, one severe problem could arise working with such a small ablation, its presence may interfere with normal hatching process in vitro since the blastocyst may be trapped or strangulated which could either result in blastocyst degeneration or monozygotic twinning (Cohen and Feldberg, 1991; Van Langendonckt et al., 2000). Since small openings will not be detectable at later developmental stages in the vast majority of cases (Abdelmassih et al., 2002) application of assisted hatching in such embryos would unintentionally create an additional opening in the zona making hatching conditions even worse.

A recent approach of Moser et al. (2004) tried to prevent this theoretical disadvantage by introducing a modified version of laser-assisted ICSI. The authors did not drill a single hole but much rather thinned the zona at the site of planned injection. Therefore, the glycoprotein matrix was levelled down to approximately 50% of the original thickness using 5–6 laser shots (6 ms) ensuring a 60–70 μm ablation. This procedure kept significantly (P < 0.01) more oocytes from leakage (94%) than routine ICSI did (88.9%). An additional advantage was a significant increase (P < 0.01) in the number of hatching blastocysts (24.3 versus 9.8%). Essentially more important, all blastocysts started to escape from the zona pellucida at the site of laser manipulation and some hatching was even detectable at premature stages (days 3 and 4) strongly indicating that multiple herniation does not occur.

To conclude, laser-assisted ICSI proved to be a reliable tool in terms of oocyte rescue during ICSI irrespective of size and shape of the ablation; however, thinning the zona more extensively prior to penetration will additionally assist and facilitate hatching in vitro.

**Assisted hatching**

Since the blastocyst has to leave the outer shell as a prerequisite for successful implantation any change of the structure of the zona pellucida will impair the hatching process per se. Regardless of whether dimension and consistence of the zona are initially different (Cohen et al., 1989; Ebner et al., 2002a) or prolonged culture in vitro may have forced those changes (Cohen et al., 1990; Schiewe et al., 1995b) it has to be ensured that the embryo has the possibility to leave the zona pellucida on day 5 or 6 to avoid the risk of necrosis. If a possible impairment of the hatching process is suspected embryologists tend to assist embryos by offering them a single artificial gap, which has been created either mechanically (Malter and Cohen, 1989), chemically (Gordon and Talansky, 1987), enzymatically (Fong et al., 1998), or with laser assistance (Tadir et al., 1991).

Out of these four different approaches the laser technique (particularly the diode laser) was found to be the easiest and fastest procedure (Balaban et al., 2002). In this retrospective study dealing with 794 cycles of bad prognosis, clinical pregnancy rate per embryo transfer varied between 46% (chemically with acid Tyrode’s) and 49.3% (partial zona dissection) but was not related to any hatching technique (implantation rate was neither). However, there is evidence from one prospective study...
of women with advanced age (Hsieh et al., 2002) indicating that in terms of implantation rate (8.2 versus 3.8%) as well as pregnancy rate (31.8 versus 16.1%) laser-assisted hatching using the 1.48 μm wavelength laser is superior at least to the chemical method (P < 0.05).

The clinical relevance of assisted hatching is still under debate and comparison of studies is difficult since prospective randomized studies are rare (Primi et al., 2004) and not all of them analysed adequate control groups. In addition, individual techniques of hatching (Table I) may cause divergences in hatching process per se and in further outcome. Indeed, a significant relation between the size of a zona opening and the completion of the hatching process in vitro has been described in an animal study on cattle blastocysts (Schmoll et al., 2003), according to which smaller holes (7–15 μm by 40 μm) caused trapping of the blastocyst to a higher degree than larger ones (40 μm by 40 μm).

Further evidence comes from a mouse model (Montag and van der Ven, 1999) clearly indicating that a certain diameter (>10 μm) is necessary for efficient hatching.

Complete hatching

However, it has to be considered that any zona manipulation will cause alterations in the hatching process (Montag et al., 2000d), especially regarding the mode and timing of it. Schmoll et al. (2003) noticed that complete hatching of the zona pellucida involving a full-thickness defect (Figure 1A) caused immediate efflux of blastocoele fluid (including tiny fragments) resulting in blastocyst shrinkage and a detectable increase in zona thickness. This phenomenon is obviously related to a drop in intracellular pressure, which is at its highest prior to rupture of the zona, and the question arises whether total hatching at earlier stages might impair natural increase of internal pressure during expansion as a prerequisite for hatching.

However, lessons learned from bovine blastocysts (Park et al., 1999) and mouse embryos (Germond et al., 1995) do not indicate a negative effect of complete hatching on the hatching process. The latter study found that significantly more (P < 0.0001) embryos started to bleb out of the zona pellucida after total opening of the zona (79.4%) compared to a untreated control group (27.4%). The same effect, e.g. similar blastulation rate but increased hatching rate, could be observed in thawed human embryos (Wong et al., 2003).

Logically, it would be expected that a higher number of hatching day 5 embryos would automatically result in an improved outcome at least in a selected group of patients with poor prognosis. Indeed, women of advanced age may benefit from prospective complete hatching as could be shown in a small (n = 48) cohort with a maternal age >35 years (Montag and van der Ven, 1999) and a larger (n = 131) but slightly older (≥38 years) patient group (Hsieh et al., 2002). Germond et al. (2000) successfully applied complete hatching to frozen-thawed embryos usually known to be related with a poorer outcome. In detail, hatching increased implantation rate from 3.6 to 14.7% and clinical pregnancy rate from 10.5 to 29.6%.

Interestingly, one study (Ali et al., 2003) concluded that only younger women (<37 years) gain profit from complete hatching; however, since not all embryos per patient in the study group were hatched the prognostic value of these data remains unclear.

Recently, a European multicenter prospective randomized study tried to assess the usefulness of the 1.48 μm diode laser in patients of poor prognosis, e.g. older than 37 years or FSH >10 IU/l, frozen-thawed cycles or implantation failure (Primi et al., 2004). The only subgroup that apparently did benefit from the laser treatment of their embryos was the one with repeated implantation failure. Though these data did not reach statistical significance they are in line with meta-analyses on the impact of assisted hatching (laser, mechanically and chemically) on conception (Edi- Osagie et al., 2003a; Sallam et al., 2003).

Partial hatching

Some authors (Baruffi et al., 2000; Petersen et al., 2002) tended to partially disrupt the zona pellucida much rather than to open it completely (Figure 1B). By doing so, two possible advantages may occur, firstly intracellular pressure can develop in a similar manner as in untreated embryos, provided that expansion of the embryo would not lead to a premature burst of the outer coat, and secondly, any theoretical harming of the embryo is prevented if the inner layer of the zona pellucida is kept intact.

Table I. Technical details of prospective studies on laser-assisted hatching

<table>
<thead>
<tr>
<th>Authors</th>
<th>Day</th>
<th>Number of laser shots</th>
<th>Irradiation time (ms)</th>
<th>Size (μm) of opening</th>
<th>Hatching mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>Montag and van der Ven (1999)</td>
<td>2</td>
<td>2</td>
<td>18</td>
<td>15–35</td>
<td>Total</td>
</tr>
<tr>
<td>Baruffi et al. (2000)</td>
<td>2</td>
<td>1</td>
<td>12</td>
<td>16–18</td>
<td>Partial</td>
</tr>
<tr>
<td>Blake et al. (2001)</td>
<td>3</td>
<td>−6</td>
<td>nd</td>
<td>80</td>
<td>Thinning</td>
</tr>
<tr>
<td>Mantoudis et al. (2001)</td>
<td>2</td>
<td>2</td>
<td>20</td>
<td>−25</td>
<td>Total</td>
</tr>
<tr>
<td>Mantoudis et al. (2001)</td>
<td>2</td>
<td>−7</td>
<td>20</td>
<td>&gt;100</td>
<td>Thinning</td>
</tr>
<tr>
<td>Ebner et al. (2002a)</td>
<td>2, 3</td>
<td>3</td>
<td>12</td>
<td>35</td>
<td>Partial</td>
</tr>
<tr>
<td>Hsieh et al. (2002)</td>
<td>3</td>
<td>nd</td>
<td>5–10</td>
<td>5–10</td>
<td>Total</td>
</tr>
<tr>
<td>Petersen et al. (2002)</td>
<td>2, 3</td>
<td>1–2</td>
<td>10</td>
<td>15–20</td>
<td>Partial</td>
</tr>
<tr>
<td>Moser et al. (2003)</td>
<td>1</td>
<td>7–10</td>
<td>6</td>
<td>−70</td>
<td>Thinning</td>
</tr>
<tr>
<td>Wong et al. (2003)</td>
<td>2, 3</td>
<td>3</td>
<td>2</td>
<td>−23</td>
<td>Partial</td>
</tr>
<tr>
<td>Wong et al. (2003)</td>
<td>2, 3</td>
<td>2–4</td>
<td>2</td>
<td>−21</td>
<td>Partial</td>
</tr>
<tr>
<td>Primi et al. (2004)</td>
<td>2, 3</td>
<td>1–4</td>
<td>12–30</td>
<td>20</td>
<td>Total</td>
</tr>
</tbody>
</table>

Size of opening corresponds to the outer ablation; nd = no data available.
A randomized controlled comparison revealed that total hatching is likely to be superior to partial hatching in terms of completion of hatching process, since the rate with the latter did not significantly differ from that of control cohort (Wong et al., 2003). Consequently, partial zona dissection did not statistically improve implantation (17.7 versus 20.8%) and pregnancy rate (33.3 versus 40.3%) in younger women (=37 years) as compared to a similar age group without application of laser (Baruffi et al., 2000). Petersen et al. (2002) suspected the same uselessness of hatching in a patient population of advanced age (≥38 years).

However, partial zona hatching proved particularly useful in a subgroup with oocytes that could hardly be penetrated by an ICSI pipette (Ebner et al., 2002a). Briefly, 96 affected cycles were randomly split up into a hatching group (n = 52) and a non-hatching group (n = 44). Both implantation rate (9.8 versus 21.7%) and clinical pregnancy rate (13.6 versus 33.6%) could at least be doubled applying partial dissection of the zona.

Theoretically, any effect on blastocyst hatching would strongly depend on the thickness of the remaining zona layer. Thus, it could happen that in certain cases the opening of the zona pellucida is not appropriate to let the embryo escape, somewhat resembling unhatched conditions.

Zona thinning

In order to circumvent a possible insufficient hatching procedure Blake et al. (2001) expanded the area of ablation up to approximately one quarter of the acellular matrix (Figure 1C). This approach positively influenced initiation (P = 0.009) and completion (P = 0.02) of the hatching process in vitro. In detail, 67.8% of the blastocysts started to herniate and another 33.9% of them completed hatching on day 7 of development, compared to 33.3 and 0%, respectively, in their untreated counterparts. The same authors tried to figure out if the actual site of hatching corresponded to the artificially thinned zona. It turned out that not a single blastocyst hatched from sites other than the laser site. For methodical reasons, some of the hatching spots were not identifiable during SEM analysis, only 16 of 48 blastocysts gave valid results, but this observation is supported by others (Moser et al., 2004). Kung et al. (2003) could show that cryopreservation of blastocysts the zona of which had been thinned extensively can lead to acceptable implantation (16.9%) and clinical pregnancy rates (31.4%).

There is only one study comparing all three different approaches to laser-assisted hatching (Mantoudis et al., 2001). It indicates that zona should rather be thinned (quarterly or partially) than totally dissecting it (P < 0.001). However, in this study complete hatching was achieved with two laser shots creating a very small opening which might have impaired hatching per se. One additional reason for the published benefit of zona thinning may be the fact that a more extensive ablation increases the chance that the artificial opening corresponds to the naturally predisposed spot of hatching (Sathananthan et al., 2003).

Biopsy

Once the zona pellucida is opened access to the perivitelline space by micropipettes is facilitated, e.g. to remove cells for PGD or to improve embryo morphology.

Usually the opening for performing any kind of biopsy was done chemically by using acid Tyrode’s (Handyside et al., 1990) or mechanically with glass needles (Verlinsky et al., 1990; Munne et al., 1995). Unfortunately, both methods face

Figure 1(A). Day 2 embryo after laser-assisted total hatching. Outer diameter of ablation is approximately 50 μm. (B) Day 2 embryo after laser-assisted partial hatching. Arrow indicates intact zona layer (8 μm). Outer diameter of ablation is approximately 45 μm. (C) Day 2 embryo after laser-assisted zona thinning. Area of thinning is approximately 80 μm.
the untrained embryologist with certain disadvantages. On one hand, acid Tyrode’s may create larger openings than wanted which may cause damage or loss of blastomeres, and on the other, any mechanical approach will have to operate with relatively sharp glass tools which may result in lysis (e.g. polar bodies).

Usage of the 1.48 μm diode laser for zona perforation guarantees standardized openings which can easily be adjusted to the actual diameter of the required biopsy pipette (which of course can be blunt-ended). Another benefit is that laser drilling as well as biopsy can be done without changing the culture dish or glass tools (in contrast to drilling with acid Tyrode’s) both of which may help to avoid contamination of probes diagnosed by sensitive techniques in PGD (e.g. PCR).

In a recent comparison, Joris et al. (2003) found an improved survival rate ($P = 0.02$) of blastomeres in laser drilling for biopsy (98.3%) as compared to acid Tyrode’s drilling (95.2%). Though implantation rates and ongoing pregnancy rates did not differ in this study application of a diode laser for biopsy was the recommended method of choice.

**Biopsy for PGD**

During preimplantation development polar body biopsy is the first possibility to analyse chromosomal constitution, at least of the oocyte. While the mechanical approach (Verlinsky et al., 1990) is performed with the polar bodies at the 6 or 12 o’clock position, zona pellucida in laser biopsy can be opened in close vicinity to the polar bodies, thus facilitating estimation of the required opening (Montag et al., 1998). In their pivotal study, Montag et al. (1998) showed that after laser-assisted polar body biopsy of mouse zygotes both polar bodies always maintained their cellular integrity, and much more importantly, it became apparent that blastocyst formation rate (88%) was unaffected compared to sham-treated (89%) and untreated (93%) counterparts, which, by the way, showed a delay in hatching as well as a decrease in the number of hatching blastocysts both indicating absence of any detrimental effect of laser usage.

In the same laboratory first application of laser-assisted polar body biopsy on humans was reported (Montag et al., 1997) and more recently these authors published their experience with polar body biopsy (aneuyploidy testing) under the restrictive German embryo protection law (Montag et al., 2004). Promising pregnancy and delivery rates, which are comparable to results from the most experienced group in Chicago, where polar body biopsy is performed mechanically (Kuliev et al., 2003), indicates that laser-assisted biopsy is another step towards an improved outcome.

The situation in vitro somewhat changes talking about blastomere biopsy on day 3 (Handyside et al., 1990) because at this stage the embryo occupies most of the space within the zona pellucida and sometimes a certain minimum distance to the blastomere cannot be maintained (Chatzimeletiou et al., 2001). One approach to avoid this possible problem could be to introduce a modified laser-drilling technique in order to ensure ample perivitelline space (Chang et al., 2004).

However, except for one study (Malter et al., 2001), laser drilling proved to be a potential alternative to conventional acid Tyrode’s drilling. Since the first pregnancy was achieved in a couple suffering from haemophilia (Boada et al., 1998) laser drilling for blastomere biopsy became a routine method in IVF laboratories (De Vos and Van Steirteghem, 2001; Han et al., 2003; Joris et al., 2003).

Although there is evidence that further in vitro development of 8-cell embryos is not impaired after removal of up to two blastomeres for prenatal genetic diagnosis (Hardy et al., 1990) concern has been raised regarding the maximum reduction of the cellular mass (Liu et al., 1993) and its possible adverse effect on the consistency of the inner cell mass. However, blastocyst biopsy (Dokras et al., 1990) circumvents the analysis of embryonic material since trophodermal cells can serve as source for PGD. In addition, at blastocyst stage more cells can be analysed with a lower percentage of cellular loss than in the 8-cell embryo.

The 1.48 μm infrared laser can also be used successfully in cases of blastocyst biopsy (Veiga et al., 1997). Therefore, zona of the blastocyst is artificially opened on the opposite side of the inner cell mass in order to avoid accidental protrusion of embryonic cells. After complete opening has been done blastocysts are kept in culture until trophectoderm starts to bleed out of the perforated zona. Thus, easy access to the trophectoderm is gained allowing for feasible dissociation of numerous trophectodermal cells by means of a biopsy pipette (de Boer et al., 2004) or laser energy (Veiga et al., 1997). If the latter method has been chosen for manipulation of the trophectodermal herniation it has to be mentioned that the laser exposure has to be approximately three times longer than for previous zona drilling (Veiga et al., 1997).

**Removal of detrimental structures**

Montag et al. (1999a) brought up another application of the 1.48 μm diode laser when he suggested to perforate the zona pellucida for removal of cytoplasmic fragments. This cosmetic micromanipulation has been introduced by Alikani et al. (1999) who tried to rescue bad quality embryos. Fragmentation in vitro is a common feature of cleaved embryos and its removal may have two positive effects; firstly, spatial relationship within the embryo is restored which is important if fragments are situated in close proximity of cleavage planes, and, secondly, secondary degeneration of blastomeres, which is thought to be causally related to cytoplasmic fragmentation (Sathananthan et al., 1990), is prevented.

Usage of a diode laser could reduce this damage due to its flexibility in hole size which can easily be adapted to the actual dimension of fragments. Nevertheless, some problems may occur during removal of fragments which are situated between blastomeres and apposed to the ablated area (Malter et al., 2001). This phenomenon was explained by the greater rigidity of the zona pellucida after laser ablation as compared with the more graded lysis effect of acid treatment.

A similar approach is the laser-assisted removal of necrotic blastomeres from cryopreserved embryos after thawing (Rienzi et al., 2002). It is well accepted that these partially damaged embryos are not as viable as fully intact ones (Van den Abbeel et al., 1997). Assuming that poor implantation rates reported after transfer of cryopreserved embryos showing one or even more necrotic blastomeres is, at least in part, caused by toxic metabolites from those affected cells, total removal of this cellular debris might improve the developmental potential of
the corresponding embryos (Rienzi et al., 2002). These authors (Rienzi et al., 2002) performed aspiration of necrotic blastomeres through a 20 μm laser-created zona opening using a 10 μm micropipette. Embryos thus corrected developed a significantly higher (P < 0.01) cleavage rate (74.3%) by 18 h of post-thaw culture as compared to thawed embryos without removal of necrotic areas (27.5%). Consequently, the observed benefit of laser-assisted removal of necrotic blastomeres on further development continued until implantation as indicated by an obvious improvement in implantation rate (16.2 versus 4.3%) and ongoing pregnancy rate (40 versus 11.4%). Increased viability in cosmetically restored embryos is further supported by relatively low rates of preclinical abortions and/or first-trimester losses (15%) as compared to data from literature (Macas et al., 1998).

To conclude, the laser-assisted removal technique proved to be a safe, easy and fast approach; however, technical problems may arise if more than two blastomeres located in different areas of the thawed embryo became necrotic (Rienzi et al., 2002).

**Hemizona assay**

In search of additional applications of the diode laser in IVF the ultimate level of dissection would be to cut the zona pellucida into two identical halves. These matching counterparts may serve as internal controls in the hemizona assay, which is a functional bioassay to test actual binding and fertilization competence of human spermatozoa (Burkman et al., 1988; Gamzu et al., 1994). Initially, zona manipulation was performed by use of microblades (Burkman et al., 1988) or manual bisection (Sanchez et al., 1995) both of which had their technical limitations, e.g. predisposition of special holding pipettes or unequal splitting. Using the InGaAsP-semiconductor laser operating with a 1.48 μm wavelength hemizona generation might be facilitated by dissecting the zona with approximately 20 laser pulses each for a duration of 20 ns (SCHÖPPER et al., 1999). The laser shots have to be applied tangentially resulting in a ring-like continuous ablation. After complete removal of the equatorial zona the gamete has to be turned by 90° followed by gentle separation of both halves using two conventional holding pipettes (Montag et al., 2000a). Obtained diameters of laser dissected hemizonae (98 ± 10 μm) were similar to those of manually bisected (107 ± 8 μm) ones (Montag et al., 2000a). Most importantly, laser application did not impair sperm binding characteristics.

**Manipulation of spermatozoa**

Though most of the laser studies have focused on the manipulation of zona pellucida in oocytes and embryos it has to be remembered that spermatozoa were the first target of laser application when sperms were trapped using continuous laser microbeams as laser tweezers (Tadir et al., 1989, 1990). In this context, König et al. (1996) warned of lasers working in the UV and near infrared range because their use may result in irreversible cell damage. For example, exposure to UV light caused paralysis of spermatozoa within 35 ± 20 s and necrosis in 65 ± 20 s. However, wavelengths above 800 nm, turned out to be less harmful.

**Paralysis of spermatozoa for cryopreservation**

Montag et al. (1999b) used this time and energy dependent effect on spermatozoa to paralyse male gametes prior to cryopreservation with the help of a diode laser. This is of special importance in patients with severely reduced sperm count or patients with surgical sperm extraction. In these men cryopreservation of the vast majority of motile sperms available would be beneficial for further cycles. Since cryopreservation in case of very low sperm count cannot be done with standard procedures due to detection problems after thawing, special containers such as cryoloops (Schuster et al., 2003), algae (Just et al., 2004), or empty zona pellucida (Cohen et al., 1997) were used. At least in the latter case problems arose since motile spermatozoa tended to swim out of the cell free zona before a sealant could be applied to the opening. Laser-assisted paralysis of the spermatozoa, as described by Montag et al. (1999b), allows for a 92% sperm recovery rate which is above the rates achieved with other methods (Cohen et al., 1997; Schuster et al., 2003). A single laser irradiation (0.5–2.5 mJ) was aimed near the middle of the sperm at a distance of approximately 15 μm which turned out to result in permanent immobilization. At lower energy levels paralysis was only temporary and motility started to restore after 2–5 min. According to the hyperosmotic swelling test and Hoechst staining data 84% of cryopreserved gametes were viable after thawing showing that integrity of the sperm membrane is not affected at low laser energy.

**Immobilization of spermatozoa prior to ICSI**

A similar experimental set-up was used to evaluate the potential of the 1.48 μm semiconductor laser for immobilization and permeabilization (as a prerequisite for successful fertilization) of human sperm prior to intracytoplasmic injection (Montag et al., 2000b). In principle, two techniques have been developed by the authors, an indirect single shot approach and a direct application at lower energy levels (double shot strategy).

The indirect technique somewhat resembles the previously described technique bringing about paralysis of spermatozoa. Similarly, only one shot was placed in close vicinity (5 μm) to the mid-tail region of the gamete. According to the energy applied different degrees of immobilization and permeabilization could be achieved. For example, if the series of experiments was done in highly viscous polyvinylpyrrolidone (PVP) a 2 mJ laser pulse was found to be sufficient for both permanent motility arrest and complete permeabilization of membranes (irrespective of the velocity of the sperm).

For the double shot strategy the first indirect laser shot applied (5 μm from mid-tail) was aimed to temporarily arrest the spermatozoon. Thus, the energy was adapted to culture conditions and the actual velocity of the sperm. In case of PVP, a laser pulse of 0.5 mJ was sufficient to cause an immediate stop (at least temporarily) of movement. The second laser shot was of lower energy (0.25 mJ) and always focused directly on the sperm tail. By splitting up energy into two separate pulses the total energy applied to the sperm could be reduced. Montag and coworkers (2000b) also showed the activation potential of spermatozoa treated with the double shot technique in mouse oocytes. However, due to legal restrictions in Germany they were not able to prove their findings on human oocytes.
These missing data could be provided by our group more recently (Ebner et al., 2001b) when human spermatozoa, twice treated with low dose laser pulses, were successfully injected into human oocytes. Not only fertilization rate (66.4%) was comparable to that of oocytes injected with mechanically treated sperms (73%), but also early cleavage rate 24h after ICSI (12.6 versus 16.3%), percentage of good quality embryos on day 2 (33.3 versus 34.8%) and blastocyst formation rate (35.6 versus 38.3%) on day 5 of in vitro development. To conclude, this alternative mode of immobilization gives good results with ejaculated as well as operatively obtained spermatozoa (Ebner et al., 2002b) and also reveals certain advantages over the conventional mechanical mode of immobilization including its usability in culture medium (Montag et al., 2000b), its non-contact mode (if sperm is not located close to the bottom of the culture dish) and its laser objective which allows for a higher magnification (Ebner et al., 2001b).

**Assessment of sperm viability**

Most recently, an additional application was introduced by Aktan et al. (2004) who used the diode laser to assess viability in cases of complete asthenozoospermia. Usually, either the hypo-osmotic swelling test (Jeyendran et al., 1984) or the mechanical touch technique (de Oliveira et al., 2004) were used in order to distinguish between viable but immotile spermatozoa and dead ones. Applying a single laser pulse (1.2 ms) at the very end of the sperm tail (direct method) caused a characteristic curling of the tail end. Since non-viable sperm did not show this phenomenon this new technique helped to identify spermatozoa with a functional integrity of its membrane.

**Conclusion**

To our experience, the 1.48 μm diode laser provides a promising tool for the microdissection of subcellular targets. In order to describe the quality and efficiency of the InGaAsP semiconductor laser in IVF laboratories three main features must be mentioned, namely the rapidity, the simplicity and the safety of the procedure.

The first two characteristics can definitely not be questioned since the almost instantaneous effect on the target structure without the help of additional devices (e.g. holding pipette) and the applicability of conventional culture dishes allows application even by untrained operators. However, the third aspect requires further discussion.

**Safety aspects**

Several animal studies, mostly on the mouse, were performed prior to switching to the human model. All of them indicate that in vitro development of mouse zygotes is not affected up to blastocyst stage, much rather the number of hatching mouse blastocysts significantly increased after zona manipulation (Rink et al., 1994a, 1996). Germond et al. (1996) investigated the further fate of laser treated animal embryos when they transferred them to foster mothers. Similar pregnancy and delivery rates as well as an unaffected sex ratio support application of this diode laser treatment to embryos from IVF patients (Germond et al., 2000).

Investigating the efficacy and safety of diode laser application for assisted hatching in humans Germond et al. (1995) analysed the zona pellucida of mouse oocytes and zygotes electron microscopically. This analysis showed that no ultrastructural changes were caused by laser-assisted manipulation of the glycoprotein matrix. However, at light microscopical level it was suspected that the choice of the laser target site may have a certain impact on cytoplasmic condition. The authors (Germond et al., 1995) found that the target site should be chosen on the equatorial plane with the laser radiation aiming tangentially and not on a more inward area with the laser beam passing the perivitelline space since in the latter case the laser could induce a local change in the appearance of the ooplasm. Rotation of the mouse gamete further ensured that this alteration spread throughout the cytoplasm along the suspected laser path. Though all observed changes were reversible (after a few hours), lysis never occurred, and normal in vitro development was seen in the mouse model, it is obligatory that for application in human the tangential irradiation has to be chosen.

In addition, it has been suspected that the absence of morphological changes under the microscope does not necessarily exclude an impairment of embryo viability due to the laser treatment (Chatzimeletiou et al., 2001). Chatzimeletiou et al. (2001) observed a damage in blastomeres next to the drilled hole by using two fluorescent markers indicating embryo viability. These authors and others (Douglas-Hamilton and Conia, 2001) suggest short pulses (e.g. 5 ms) at lower energy levels in order to prevent any theoretical drawback of laser usage. For the mouse, a minimal working distance of 8 μm to the nearest blastomere has been recommended (Chatzimeletiou et al., 2001) which avoided any harm to the cells. It seems logical that due to the large perivitelline space this safety distance can easily be maintained at earlier cleavage stages, whereas it may be a problem at later stages, as indicated by the immediate reaction of blastocysts to laser pulses (blebbing out of trophectodermal cells, reversible embryonic collapse). This observation would be a strong argument for partial zona hatching or zona thinning instead of total hatching. In addition, it seems reasonable to suppose that total opening of the zona for biopsy on day 3 should be performed on day 2 at a time any recommended safety distance can be maintained easily.

Laser-assisted treatment of spermatozoa for any purpose is something completely different since energy levels can be reduced by a factor 10 compared to zona manipulation (Primi et al., 1999). Depending on the optical equipment of the laboratory 2–3 mJ were sufficient to immobilize and permeabilize human spermatozoa (Montag et al., 2000b) without affecting the oocyte activation and fertilization potential (Montag et al., 2000b; Ebner et al., 2001b). The total energy dose a spermatozoon is exposed to can be further reduced if the double shot technique of Montag et al. (2000b) is applied. However, both shots are aimed far from the sperm head containing the genetic material. Moreover, Montag and Rink (2001) did not find an increase in DNA fragmentation even after they applied multiple shots directly on the sperm head, probably due to the minimal damage zone (1 μm) of the diode laser beam (Germond et al., 1995) and the fact that DNA in mammalian sperm is tightly compacted into linear arrays organized as loop domains (Ward and Coffey, 1991).
To summarize, though almost a double energy level is required for adequate ablation of human zona pellucida compared to mouse counterparts, it is still within a range at which no thermal effects have been observed in mouse oocytes (Germond et al., 1995). Nevertheless, it may be advisable that every laboratory develops its own diode laser set-up in order to find the minimal energy value, which ensures an adequate effect on the zona pellucida and/or spermatozoon. The harmlessness of the 1.48 μm diode laser is further supported by publications which report the birth of healthy offspring.

Outcome after diode laser application

A report on the impact of assisted hatching with different techniques has recently been published (Edi-Osagie et al., 2003b). In this meta-analysis only 6 of 23 trials included reported live birth data. Based on this limited data no positive effect of assisted hatching on take-home baby rate could be seen. On the other hand, all techniques may be considered as safe since no adverse effects on perinatal and obstetric outcome could be noted. The same will hold for the diode laser as well, since animal as well as human data are promising. In a study on mice, an inconspicuous anatomical situation was found and the reproductive ability of a F2 mouse generation could be shown (Germond et al., 1996). Preliminary data reporting the first births and indicating no negative influence of diode laser-assisted hatching (Germond et al., 2000) were recently supported by other authors who focused on the rate of chromosomal aberrations and congenital malformations in 134 children born after this treatment (Kanyo and Konc, 2003). Though no control group was used in the latter study rates of de-novo chromosomal alterations (0%) and major malformations (2.2%) were within a normal range.

Similarly, successful births have been reported for almost all above mentioned applications. Rienzi et al. (2001) were the first to achieve a pregnancy and delivery of three babies after laser-assisted ICSI. Acceptable take-home baby rates for laser-assisted polar body biopsy have been published (Montag et al., 2004). Furthermore, a successful case report can be found in literature using diode laser for embryo biopsy (Han et al., 2001). In addition, laser-assisted manipulation of human spermatozoa prior to ICSI led to the birth of a healthy girl (Ebner et al., 2002c).

To conclude, in times when patient numbers continue to grow, the introduction of alternative methods (e.g. diode laser), making laboratory work simpler and quicker but without affecting quality of the work should be appreciated and may be of considerable benefit. However, it is strongly emphasized that safety aspects have to be kept in mind when introducing a new technique.

References


Received on November 24, 2004; Revised on January 26, 2005; Accepted on January 27, 2005.