Improved fertilization and implantation rates after non-touch zona pellucida microdrilling of mouse oocytes with a 1.48 µm diode laser beam

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The safety of microdrilling the zona pellucida of mouse oocytes with a 1.48 µm diode laser has been investigated by determining the ability of mouse oocytes to fertilize *in vitro* and develop *in vivo*. Mice born after transfer of control and zona pellucida-microdrilled embryos into foster mothers were submitted to anatomical and immunohisto-chemical investigations, and their aptitude to breed was assessed in two subsequent generations. Decoronization of the oocytes with hyaluronidase induced a reduction of the fertilization and implantation rates, which was attributed to a zona hardening phenomenon. After laser zona pellucida microdrilling, these rates were restored to those obtained with embryos derived from untreated oocyte–cumulus complexes. Pups derived from zona pellucida microdrilled embryos were comparable with those obtained from control embryos, confirming the lack of deleterious effects of the laser treatment. In conclusion, the 1.48 µm diode laser allows safe microdrilling of the zona pellucida of mouse oocytes after decoronization with hyaluronidase. Based on the health of the F_2 generation and the lack of neuroanatom-ical and neurochemical differences, we concluded that this technology may be investigated in the human, particularly when the zona pellucida represents the main impediment for fertilization or embryo hatching.

Key words: assisted reproduction/infrared diode laser/micro-drilling/mouse/zona pellucida

Introduction

Chemical (Gordon and Talansky, 1986; Garrisi *et al.*, 1990), mechanical (Depypere *et al.*, 1988; Malter and Cohen, 1989; Odawara and Lopata, 1989) or laser methods (Feichtinger *et al.*, 1992; Laufer *et al.*, 1993; Schütze *et al.*, 1994, 1995) have been used to produce holes in the zona pellucida of mammalian oocytes in order to improve fertilization (Gordon, 1988; Gordon *et al.*, 1988; el Danasouri *et al.*, 1993; Ng *et al.*, 1993; Enginsu *et al.*, 1995), facilitate blastocyst hatching (Cohen *et al.*, 1990; Gordon and Dapunt, 1993; Obruca *et al.*,

1994; Neev *et al.*, 1995; Schiewe *et al.*, 1995) or perform blastomere biopsy (Tarín and Handyside, 1993). Recently, an infrared 1.48 µm diode laser light, focused through a micro-scope objective, was shown to allow rapid, easy and non-touch microdrilling of mouse and human zona pellucida while maintaining a high degree of accuracy under conventional culture conditions (Rink *et al.*, 1994). The drilling effect was shown to be due to a highly localized heat-dependent disruption of the zona pellucida glycoprotein matrix (Rink *et al.*, 1996), a phenomenon markedly different from the photo-ablation induced by wavelengths close to the UV region (Neev *et al.*, 1992, 1993). Embryos could be maintained in their usual culture dish and medium during the drilling process without requiring special quartz optical equipment as for UV lasers (Schütze *et al.*, 1994), a change of medium (Blanchet *et al.*, 1992) or micromanipulators as for the 2.9 µm Erbium:YAG laser (Obruca *et al.*, 1994). Contrary to the detrimental effect on precompacted mouse embryos (Schiewe *et al.*, 1995) induced by the 308 nm xenon-chlorine excimer laser (Neev *et al.*, 1993), the drilling process in the infrared region did not affect embryo survival in mice (Germond *et al.*, 1995; Rink *et al.*, 1996) or in humans (Antinori *et al.*, 1994). The purpose of this study was to obtain additional proof that the infrared 1.48 µm diode laser could be used safely for human applications. For this purpose, the capacity of mouse oocytes to fertilize and implant after laser zona drilling was studied, the reproductive ability of the pups derived from the procedure tested, and the lack of anatomical or immunohistochemical alterations in the progeny checked.

Material and methods

Mouse oocytes and spermatozoa

Female mice (B6D2F1; IFFA, Credo, France) aged 5–8 weeks were stimulated (day 1) with one peritoneal injection (10 IU/0.2 ml) of follicle stimulating hormone (FSH, Folligon; Intervet AG, Pfäffikon, Switzerland), followed on day 3 by a second injection (10 IU/0.2 ml) of human chorionic gonadotrophin (HCG, Pregnyl; Organon, Zürich, Switzerland) to induce ovulation. Females were killed by cervical dislocation 13 h after HCG administration. The swollen ampullae of the oviducts were dissected; the available oocyte–cumulus complexes were isolated under a stereo microscope, suspended in an insemination medium consisting of Whittingham's medium (Whittingham, 1971) supplemented with 3% bovine serum albumin (BSA, Cat. No. A-3311; Sigma, St Louis, MO, USA), and stored immediately under standard incubation conditions (37°C, 5% O_2, 5% CO_2, 90% N_2).

Spermatozoa were squeezed out of the cauda epididymis of adult males (B6D2F1) mated 1 week earlier, and allowed to disperse and capacitate in the insemination medium for 90–120 min.

In-vitro fertilization

The available oocyte–cumulus complexes were obtained from 210 mice and randomly separated into three equally-sized groups: control 1, two or three oocyte–cumulus complexes were placed into 0.9 ml insemination medium in 4-well culture dishes (Nunclon, Gibco, Basel, Switzerland); control 2, two or three oocyte–cumulus complexes were dispersed with hyaluronidase (100 IU/ml) for 1–2 min and the intact metaphase II oocytes washed twice with insemination medium; laser-drilled group, intact hyaluronidase-treated metaphase II oocytes were submitted to the zona pellucida laser drilling procedure. The three groups were inseminated at the same time with suboptimal sperm concentrations of either 25 000 or 50 000 spermatozoa/ml. These concentrations were chosen because they usually give fertilization rates of ~50%, thus enabling external conditions, such as decoronization or zona pellucida drilling, to measurably affect the fertilization rates.

Microscopic evaluation of fertilization

The oocytes were washed free of spermatozoa 6–8 h after insemination in INRA Ménézo B2 medium (Laboratoire CCD, Paris, France). Oocytes that had extruded the second polar body were isolated under a stereo microscope (×40, Leica, Heerbrugg, Switzerland) and observed under an inverted microscope equipped with Hoffman modulation contrast (×200, Olympus, Zürich, Switzerland) in order to assess the presence of two polar bodies and two pronuclei.

Parthenogenetic activation induced by the decoronization procedure was checked by incubating oocytes sampled from the three groups in presence of heat-immobilized (60°C, 30 s) spermatozoa. After 8 h, eggs that had extruded a second polar body and/or were expressing a nuclear structure were considered to be parthenogenetically activated and were counted.

Experimentally induced parthenogenetic activation

The calcium ionophore calcimycin (A23187, Sigma) was dissolved in 100% dimethylsulphoxide (DMSO, Sigma) and then diluted with B2 medium to a final concentration of 0.25% DMSO. Decoronized oocytes were incubated for 15 min in Ménézo B2 medium, containing 0.25% DMSO with or without 10 µM calcimycin. The oocytes were then washed four times in fresh B2 medium and cultured for 2 more days. Parthenogenetic activations were recorded at 12, 24 and 36 h after exposure to the ionophore.

Embryo transfer

The available fertilized oocytes in the three groups (control 1, control 2 and laser treated) were cultured in Ménézo B2 medium for 16–18 h. A total of 16 2-cell embryos were selected from among the cleaved zygotes in the three groups and transferred into the left oviduct of pseudo-pregnant foster mothers (Hogan *et al.*, 1986). The transfers of the three groups of embryos were performed in parallel in order to avoid interference of uncontrolled experimental conditions. As we were interested primarily in determining the viability and growth of the offspring, all pregnancies were allowed to proceed to term. The deliveries were monitored as closely as possible in order to avoid errors due to the fact that some mothers may eat some of their progeny. In three cases, a Caesarean section was performed when the delivery was delayed by more than 24 h; the pups were then entrusted to adoptive mothers whenever possible. The percentage birth rates were calculated as the number of mice born/embryos transferred.

F_2 generation follow-up

The pups were weaned from their mothers 3 weeks after birth and separated according to their phenotype and sex. Two males and two females were selected from among the pups derived from the three group of embryos. At 8 weeks of age, selected sibling mice from each group were allowed to mate in order to check their reproductive capacity. The F_2 generation obtained were grown to the adult stage and again breeding between siblings was allowed to occur.

Anatomical and immunohistochemical evaluation

At the age of 15 weeks, two males and two females were isolated from among the F_2 generations deriving from the control 1 and laser-treated groups, from in-vivo fertilized embryos (B6D2F1×B6D2F1) from embryos transferred into foster mothers (control 3) and from spontaneous breeding of the B6D2F1 strain (control 4). These mice were submitted to an anatomical and immunohistochemical evaluation.

Mice were deeply anaesthetized with Nembutal (i.p., 60mg/kg), and perfused transcardially with 100 ml of fresh 4% phosphate-buffered paraformaldehyde. Visceral organs were collected for macroscopic examination. The brain was removed from the skull, cryoprotected in 30% glucose and cut coronally on a freezing microtome in alternate series of 50 µm thick sections. One series was stained with Toluidine Blue (cellular stain). Alternate series were reacted with antibodies against the following neuronal specific markers: parvalbumin, calbindin, S-100β and calretinin (Swant, Bellinzona, Switzerland), serotonin (Incstar, Stillwater, MI, USA), tyrosine hydroxylase (Eugene Tech., Allendale, NJ, USA), γ-aminobutyric acid (GABA)-a receptor subtype α1, α2, β2/3 (gift from Dr J.M. Fritschy, Zürich, Switzerland), alpha-amino-3-hydroxy-5-methylisox-azole-4-proprionic acid (AMPA) glutamate R1 and R2/3 receptors (Chemicon, Temencula, CA, USA), glial fibrillary acidic protein (Dako, Glostrup, Denmark) or were stained histochemically for the localization of acetylcholinesterase activity (Tago *et al.*, 1986). Primary antibodies were detected with secondary biotinylated antibodies (Jackson, Westgrove, PA, USA) and revealed with the Vectastain Elite avidin/horse-radish peroxidase complex (Vector, Burlingame, CA, USA), according to the manufacturers' recommendations. The specificity of the primary antibodies has been previously assessed by the manufacturers. Omitting the primary antibody resulted in complete disappearance of the immunoreaction product.

Zona drilling

The set-up used for zona pellucida microdrilling has been described in detail elsewhere (Rink *et al.*, 1994; Germond *et al.*, 1995). Briefly, a 670 nm diode laser aiming beam and the collimated 1.48 µm cw laser beam (InGaAsP diode laser; Alcatel/Alsthom Research, Paris, France) were fed into an inverted microscope through several mirrors and focused by the microscope objective (×45) within the microscope field in a spot 8 µm in diameter. The power routinely available at the image plane of the objective was 47 mW, corresponding to a maximal power density of 94 kW/cm^2.

The oocytes were suspended in groups of 10–15 in a 4-well multidish (Nunclon) in 0.5 ml insemination medium under 100 µl mineral oil (Light mineral oil; Fisher, Fairlawn, USA). Using the X-Y microscope stage, each oocyte was positioned to bring a region of the zona pellucida on the aiming spot and the zona pellucida was exposed to 10–15 ms laser light. Depending on the irradiation time and the temperature of the suspending medium, the diameter of the drilled holes varied between 5–10 µm.

Statistical analysis

Statistical analysis was performed by calculating the χ^2 with a contingency table (StatView Student for Macintosh, Abacus Concept, Berkeley, USA).

Table I. Percentage fertilization rates (zygotes/oocytes) observed in the three groups of inseminated oocytes: control 1, intact oocyte–cumulus complexes; control 2, hyaluronidase decoronized oocytes; treated, hyaluronidase decoronized and zona pellucida laser-drilled oocytes

Sperm concentrations (10^3/ml)	Control 1	Control 2	Treated
25	106/279 (38.0)	71/287 (24.7)	182/349 (52.1)
50	843/1506 (56.0)	572/1452 (39.4)	959/1411 (68.0)

Percentages, compared by contingency table analysis, were statistically different ($P < 0.001$) when the groups or the concentrations were compared two by two.

Results

Effect of the sperm concentration on fertilization, with or without zona drilling

The early steps of the fertilization process were followed in three groups (control 1, control 2, and laser-drilled) by observing microscopically the inseminated oocytes after 1, 3 and 6 h. In control 1, the initial interaction between the gametes was difficult to observe due to the presence of the cumulus cells, but by the end of the incubation period the oocytes were totally free of corona cells and the fertilization could be assessed without additional mechanical decoronization. After hyaluronidase treatment (control 2, laser-drilled group), spermatozoa were seen to bind to the zona pellucida in the minutes following insemination. In the zona pellucida microdrilled oocytes, four different situations were observed: (i) no spermatozoon in the perivitelline space (PVS), but spermatozoa attached to the zona pellucida; (ii) a single spermatozoon swimming around the oolemma in the PVS; (iii) a spermatozoon bound to the oolemma close to the hole, and with the tail protruding out of the hole; and (iv) rarely, more than one spermatozoon in the PVS.

In control 1, the fertilization rate was increased from 38.0 to 56.0% when the sperm concentration was increased from 25 000 to 50 000/ml (Table I). Decoronization (control 2) induced a significant ($P < 0.001$) decrease in fertilization from 38.0 to 24.7% and from 56.0 to 39.4% for both sperm concentrations. When the zona pellucida of decoronized oocytes was drilled, the fertilization rates were dramatically increased ($P < 0.001$) from 24.7 to 52.1% and from 39.4 to 68.0%, to reach higher values than those observed for intact oocyte–cumulus complexes (control 1, Table I).

Polyploidy was not routinely observed but has been noted occasionally with both sperm concentrations (data not shown). Parthenogenetic activation rates induced by decoronization or drilling procedure remained <7% (Table II). In contrast, calcimycin produced parthenogenetic activation in both control (59%) and zona pellucida drilled oocytes (55%), while DMSO alone had almost no effect (2%).

Impact of laser zona pellucida drilling on in-vivo development

A summary of the embryo transfer procedure is presented in Table III. The number of pregnant foster mothers was not statistically different in the three groups. However, a significantly lower delivery rate was observed for embryos obtained

Table II. Number of oocytes undergoing parthenogenetic activation (%) per number of oocytes treated, sampled from the three groups: control 1, intact oocyte–cumulus complexes; control 2, hyaluronidase decoronized oocytes; treated, hyaluronidase decoronized and zona pellucida laser-drilled oocytes

| Groups | Parthenogenetic activation (%) | | | |
	Without spermatozoa	Heat-inactivated spermatozoa (60°C)	+ 10 μM calcimycin in DMSO	+DMSO
Control 1	2/28 (7.1)	4/21 (19.0)	–	–
Control 2	2/43 (4.7)	3/45 (6.7)[a]	23/39 (59.0)[a]	0/41 (0.0)
Treated	2/40 (5.0)	0/40 (0.0)[b]	27/49 (55.1)[b]	1/50 (2.0)

DMSO = dimethylsulphoxide.
[a,b]Values with superscripts are significantly different ($P \leqslant 0.0001$).

Table III. Pregnancy and delivery rates (mice born/embryo transferred) (%) observed after transfer of embryos derived from the three groups of oocytes: control 1, intact oocyte–cumulus complexes; control 2, hyaluronidase decoronized oocytes; treated, hyaluronidase decoronized and zona pellucida laser-drilled oocytes. The sex ratios were established among the adult live F_2 hybrids obtained

Groups	Pregnancies/transfer (%)	Mice born per embryo transferred (%)	Sex ratios male/female (%)
Control 1	4/5 (80.0)	26/80 (32.5)[a]	12/14 (46.2)
Control 2	5/8 (62.5)	20/128 (15.6)[a]	11/9 (55.0)
Treated	4/6 (66.7)	28/96 (29.2)	13/15 (46.4)

[a]Values were significantly different ($P \leqslant 0.0001$).

from decoronized oocytes ($P \leqslant 0.0001$). After laser drilling the rate was restored to that of the untreated oocyte–cumulus complex (control 1) group. The sex ratios were similar in all groups.

F_2 generation follow-up

The sex ratios in the F_2 generation were similar in the three groups and close to 50% (Table III). The reproductive ability of the F_2 generation was shown to be similar to that observed in a previous study with a mean number of 8.5, 7.5 and 8.0 pups obtained in the three groups of embryo treatment.

Anatomical and immunohistochemical evaluation

Upon macroscopic examination, the components of the digestive tract and the urinary and reproductive systems revealed no morphological alteration in the animals derived from laser microdrilled embryos as compared to those in the control groups 1, 3 and 4. The microscopic examination of the cellular organization of the brain was performed on Nissl-stained (Toluidine Blue) coronal sections of the brain and spinal cord. The laminar structure of the cerebral and cerebellar cortices as well as the size and location of the main nuclei and tracts within the brainstem and spinal cord were investigated. The major cyto-architectonic features of the mouse brain, including the rodent-specific barrelfield of the somatosensory cortex, were preserved in all animals. The immunocytochemical and histochemical analysis of the organization of the major neurotransmitter systems (glutamatergic, GABAergic, monoaminergic and cholinergic) confirmed that no apparent modification

of the expression of the specific markers (neurotransmitters, receptors, synthesizing or degrading enzymes, related proteins) occurred in the experimental animals in comparison with the controls. The parameters measured were the dendritic morphology and distribution of the labelled neuronal and glial cells, as well as the distribution and density of the axonal projections. The investigation was primarily centred on the following structures: cerebral cortex, hippocampus, basal forebrain, thalamus, hypothalamus, monoaminergic brainstem nuclei, cerebellum, and spinal cord.

Discussion

In the experiments described, dispersion of the egg cumulus by hyaluronidase was associated with significantly lower fertilization and implantation rates. It has been postulated that the cumulus–corona radiata complex participates in the selection and acrosome reaction of the spermatozoa, leading them to bind and penetrate the oocytes (Carrell *et al.*, 1993). From this point of view, the reduced fertilization rates observed after cumulus dispersion can be explained. However, this reduction cannot be associated with a decreased ability of the decoronized oocytes to bind the spermatozoa, as no differences were noticed in this respect between control 2 and laser-drilled oocytes. It thus appears that specific modifications of the zona pellucida induced by the hyaluronidase treatment are responsible for the decrease in fertilization.

Such changes, which could be called hardening, have been observed in hamster eggs after hyaluronidase treatment (Drobnis *et al.*, 1988), in mouse and rat eggs during in-vitro ageing (Zhang *et al.*, 1991; Fukuda *et al.*, 1992), after exposure to specific monoclonal antibodies (Garcia Framis *et al.*, 1994) or freeze–thaw procedure (Carroll *et al.*, 1990). Two parameters which can be used to quantify this hardening effect are a reduced fertilization rate and an increased resistance to α-chymotrypsin digestion (Eppig *et al.*, 1992). Our results show that this effect is localized in the zona pellucida, as a fertilization rate comparable to that of untreated oocyte–cumulus complexes is obtained when the spermatozoa are allowed to reach the PVS through the microdrilled hole. With the sperm concentrations used in this study, which are 20–40 times lower than those known to be optimal in the mouse, this phenomenon occurs rapidly after insemination, without a time-dependent increase in the number of spermatozoa inside the PVS. An explanation for this could be that among the cohort of the spermatozoa initially attracted by the zona pellucida, one will reach the oocyte in front of the hole and be selected to freely swim through it. The other spermatozoa remain bound to the zona pellucida and continue to compete for a chance to fertilize. Another possible interpretation would be that the spermatozoa which were able to swim through the hole were acrosome-reacted, which would explain why these spermatozoa are able to fuse with the oolemma.

No attempts were made to increase the number of acrosome-reacted spermatozoa with calcimycin (Kobayashi *et al.*, 1992) for two reasons. Firstly, we wanted to avoid ionophore-induced parthenogenetic activation of the oocytes and secondly, a high fertilization rate (68%) was obtained without it (Table I).

After transferring the in-vitro fertilized oocytes to foster mothers, a significant decrease in the birth rate was observed in the cumulus-dispersed group (control 2). The pregnancy rate in the three groups of eggs was similar, which indicated that the transfer conditions were reproducible. Similar to the situation described above for fertilization, the drilling procedure restored a birth rate close to that of untreated control eggs. Thus, the hyaluronidase treatment of metaphase II oocytes seemed to induce a hardening effect of the zona pellucida leading to reduced fertilization and possibly to impaired or delayed mechanical hatching, with the consequence that the embryos fail to implant.

As the birth rates obtained after the transfer of decoronized and laser-drilled, in-vivo fertilized zygotes have been shown to be comparable in a previous study (Germond *et al.*, 1995), one might suspect that the hyaluronidase treatment or some other undefined in-vitro conditions induces zona pellucida hardening in unfertilized metaphase II oocytes and not in zona pellucida-reacted fertilized zygotes. It is not clear to what extent the hyaluronidase treatment of oocytes prior to intracytoplasmic sperm injection (ICSI) also induces zona hardening in human eggs, as shown here in the mouse. The high implantation rates observed for ICSI-derived embryos (Palermo *et al.*, 1993) contradicts this hypothesis, but one should bear in mind that the mechanical piercing of the zona pellucida during injection might assist hatching unintentionally.

The F$_2$ generation follow-up of the mice born after transfer did not show noticeable differences among the three tested groups in terms of phenotypes and sex distribution, or of their ability to reproduce. In order to identify possibly more subtle changes induced by the procedure, mice from the control and experimental groups were submitted to anatomical and morphological examination. Numerous behavioural or motor deficits following alteration of brain development are associated with structural modification of the cerebral or cerebellar cortices (Sotelo, 1980; Hoffarth *et al.*, 1995). In addition, modifications in specific neurotransmitter systems have been shown to be related to neurological pathologies [e.g. GABA with epilepsy, catecholamines with motor and cognitive functions, acetylcholine with memory (McGeer *et al.*, 1987)]. A trophic role for early expression of neurotransmitters in embryonic brains has also been recently demonstrated (Lauder, 1993; Zhou *et al.*, 1995). The morphological and neurochemical features investigated were selected for their relevance in a number of brain development disorders. Although subtle modifications of the brain could have remained undetected by our investigations, no alteration was found using techniques suitable to identify known morphological and neurochemical changes in other animal models.

Lasers operating close to the UV region are likely to induce undesirable mutagenic side effects (Ashwood-Smith *et al.*, 1996). With the 1.48 μm infrared laser wavelength used in this study, the energy delivered at the zona pellucida of mouse zygotes was shown to have no effect on their development *in vitro* and *in vivo* (Germond *et al.*, 1995). The drilling mechanism is the result of a thermal effect induced at the focal point due to the absorption of the laser energy by water and/or zona pellucida macromolecules, leading to a confined

thermolysis of the zona pellucida matrix (Rink *et al.*, 1996). The risks of invoking direct or indirect genetic damage are likely to be low as metaphase II oocytes, extremely sensitive to external influences, retained their full ability to be fertilized, to cleave, to implant and to give rise to fertile progeny after zona pellucida microdrilling. Despite the fact that normal fertilization rates were obtained with very low sperm concentrations in the mouse, the method might not prove as successful in the human, especially when considering the high fertilization rates achieved by ICSI (Palermo *et al.*, 1993). However, further developments of our laser zona pellucida drilling approach associated with cell trapping (Enginsu *et al.*, 1995) and sperm fusion is still an unexplored alternative for mechanical insertion of the spermatozoon inside the cytoplasm, but use of these techniques for human assisted fertilization clearly demands more experimental data showing absence of damage at the nuclear and mitochondrial DNA levels.

In conclusion, the infrared 1.48 μm laser wavelength allows the drilling of holes in the zona pellucida of mouse metaphase II oocytes without affecting negatively their ability to be fertilized and to develop *in vitro* and *in vivo*. The observed enhanced implantation rate after zona pellucida microdrilling in the mouse suggests that the described procedure may be safely applied to a genetically more stable human material, such as the cleaved embryo, in order to assist hatching when the zona pellucida is suspected to interfere negatively with the implantation process.

Acknowlegements

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