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The efficacy and safety of zona pellucida drilling by a 193-nm excimer laser*

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Objective: To examine the efficiency of argon fluoride excimer laser drilling of the zona pellucida of mouse oocytes in improving in vitro fertilization (IVF) at low sperm concentrations and to assess its safety.

Design: Oocytes obtained from (Balb/c × C57BL6)CB_6F_1 female mice were drilled by laser and divided into two groups: group I (89 oocytes) were inseminated with 10^5 sperm cells/mL, and group II (94 oocytes) were inseminated with 10^6 sperm cells/mL. Both groups' fertilization rate and development in vitro was compared with control oocytes that underwent the same preparation steps but no drilling (94 and 88 oocytes for group I and group II, respectively).

Main Outcome Measures: The fertilization rate and the development in vitro of the laser-drilled groups is compared with that of the control. In addition, in vivo development of embryos generated from laser-drilled oocytes after transfer to pseudopregnant recipients is assessed.

Results: For both sperm concentrations, laser drilling significantly enhanced fertilization over control (67% versus 31% at 10^5 sperm cells/mL and 90% versus 54% at 10^6 sperm cells/mL). The development into the blastocyst stage after 96 hours of incubation was similar for both the laser-drilled and control groups at any sperm cell concentration. However, complete hatching at this point was significantly enhanced by the drilling procedure. Normal litters were obtained from the transfer of embryos developed from zona-drilled oocytes into pseudopregnant recipients.

Conclusions: Excimer laser drilling enhanced IVF at low sperm cell concentration. The procedure is safe and did not interfere with embryo development in vitro or in vivo.

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Key Words: Micromanipulation, in vitro fertilization, zona drilling, excimer laser, laser drilling, mouse oocytes

The zona pellucida (ZP) presents a barrier to sperm penetration. It has been shown that production of a gap in the ZP facilitates the passage of sperm to the ooplasm and improves the efficacy of in vitro fertilization (IVF) in animals (1–4) as well as in humans (5–10). The same technique is currently employed to assist the hatching of human embryos with unthinned zonae (11, 12) and for human embryo biopsy (13). There are two main methods to create holes in the ZP: chemical dissolution by acid Tyrode's solution or chymotrypsin (1) and mechanical partial zona dissection (8) or slitting (10). When mechanical or chemical drilling was performed in mouse oocytes that were inseminated in vitro with normal sperm, the fertilization rate and development to the blastocyst stage were similar

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to that of untreated oocytes (2, 4, 6). Infertile mice with morphologically abnormal sperm could be restored to fertility by zona drilling and produce normal offsprings (1). Furthermore, when embryos generated from drilling were transferred to pseudopregnant females, a comparable percentage of normal litters was achieved as in controls (2, 3). In humans, the application of mechanical zona dissection for enhancement of fertilization resulted in pregnancies and births of normal babies (7, 8).

Human oocytes seem, however, to differ from those of the mouse in their vulnerability to chemical micromanipulation. Garrisi et al. (14) compared the two technical approaches of creating a gap in the ZP to assist human fertilization in cases of severe male infertility and found a significant increase in oocyte damage when acid Tyrode's solution was employed and that embryonic development was compromised after chymotrypsin application. In contrast to chemical drilling, mechanical zona dissection did not result in a high rate of oocyte damage and did not affect embryonic development.

The main drawbacks of mechanical drilling are its relative technical complexity and the inability to produce standardized and uniform holes. The current micromanipulation methods employ varying degrees of sheering forces induced by the pipette and consequently result in large openings of different sizes (15). A slit that is too large may increase the rate of polyspermic fertilization (14), resulting in the extrusion of the oocyte from the zona (15) and in blastomere leakage (12, 15). On the other hand, a hole that is too small may result in blastocyst trapping if the zona does not thin after micromanipulation (12).

In an effort to overcome these problems, we have developed a unique laser methodology to produce extremely precise and reproducible holes in the ZP without detectable heating (16). The laser employed is the argon fluoride excimer laser with a 193-nm wavelength, the shortest available laser wavelength that can propagate in air. Excimer lasers are pulsed gas lasers that use a mixture of inert gases and a halogen gas to generate short (10 to 20 ns) pulses of high-energy light in the ultraviolet (UV) part of the spectrum. When pulsed UV laser radiation interacts with the surface of an organic polymer, the material is etched to a submicrometer depth per pulse by photodisruption of molecular bonds (17). The main features of this phenomenon that distinguish the interaction of UV laser pulses from visible or infrared laser pulses are the submicrometer control that can be exercised over the depth of etching and the lack of thermal damage to the substrate. The extremely precise nonthermal ablative quality of the argon fluoride excimer laser has been recently applied in human corneal keratoplasty (18).

In a preliminary work employing mouse oocytes we demonstrated the ability of argon fluoride excimer laser to create well-circumscribed, reproducible holes of desired dimensions with sharp edges. Oocytes drilled by this new method were fertilized in vitro and developed to the blastocyst stage in a rate comparable with that of a control group (16). This work was undertaken to evaluate the efficacy of laser drilling in enhancing fertilization at low sperm concentrations and assess its safety by evaluating in vitro and in vivo mouse embryo development after laser treatment.

MATERIALS AND METHODS

The Drilling System

The drilling system used in these experiments was described previously (16). In short, a model 103 MSG, Lambda Physic Laser (Gottingen, Germany) argon fluoride excimer laser with a 193-nm wavelength and pulse duration of approximately 15 ns was used. The absorption depth of 193 nm radiation by biological materials is extremely small (approximately 1 μm) (17); therefore, with each pulse of the laser a thin layer of material may be removed. This characteristic of the laser-tissue interaction enables very precise control of the drilling process. The laser beam was directed by a series of mirrors and a long focal (f_L = 1,000 mm) lens through the large end (0.5 mm in diameter) of a glass micropipette placed in an aqueous media (Fig. 1). Because of the high absorption of laser radiation by liquid, the pipette was brought to near contact with the ZP, and the

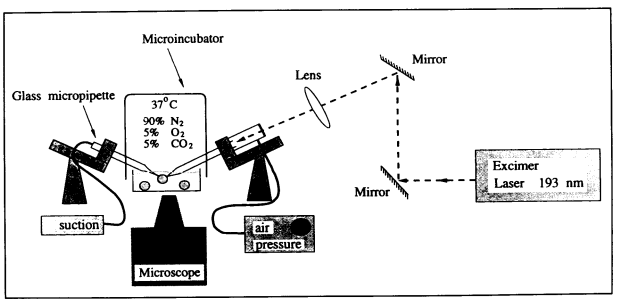

Figure 1 Diagrammatic representation of the argon fluoride excimer laser-drilling system.

liquid was pushed out of the pipette by application of positive air pressure inside the pipette so that the air/liquid border was maintained in very close proximity (approximately 1 μm) to the pipette tip. Three to four pulses of the laser beam with an energy fluency at the tip end of approximately 70 mJ/cm^2 were required until the ZP was penetrated to form a hole. Complete ablation of the ZP is recognized by a sudden enlargement of the perivitelline space and the ability of the pipette's tip to cross through the hole to this space. The spot size was determined by the diameter of the pipette tip. The total energy per pulse that emanated from an 8-μm pipette tip was approximately 30 nJ.

The alumina silicate pipettes used in these experiments were pulled from glass capillaries with a 1-mm OD and a 0.58-mm ID (Sutter Instrument Co., Novato, CA). These pipettes were pulled to a tip diameter of 8 μm by a Flaming/Brown Micropipette Puller, model p-80/PC (Sutter Instrument Co.). The energy fluency measurements were accomplished with a Laser Power/Energy Meter model DGS with a model 03AP head (Ophir Optronics Ltd., Jerusalem, Israel). Drilling was performed in a drop of T6 medium (19) containing 0.4% bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO) and supplemented with sucrose 2.57 g/dL. The dish was placed on a microscope heating stage (37°C) and covered with a chamber with an incubator-like microenvironment (90% N_2, 5% CO_2, 5% O_2). After laser drilling, both drilled and control oocytes were inseminated and cultured as described.

Oocyte and Sperm Cells Recovery

Sperm cells and oocytes were obtained from gray coat (C57Black6 × BALB/C) F1 hybrid mice as previously described (16). Briefly, superovulation was induced in 5- to 8-week-old females by injection of 10 IU IP of pregnant mare serum gonadotropin (Gestyl; Organon, Oss, The Netherlands) followed 48 hours later by 10 IU of hCG (Pregnyl; Organon). On the morning after hCG injection, animals were killed and oocytes were recovered from the ampulae. Corona and cumulus were removed using hyaluronidase (H3506, Sigma Chemical Co.) 1,000 U/mL in M2 medium (19) enriched by 0.4% BSA medium, and the oocytes were rinsed thoroughly two times in M2 medium.

Epididymides were collected from 10- to 16-week-old F1 hybrid males, and spermatozoa were recovered into T6 medium by pressing each epididymis with a pair of forceps. The spermatozoa were allowed to disperse in T6 medium for 30 minutes in an incubator (37°C, 90% N_2, 5% CO_2, 5% O_2).

Manipulated and control oocytes were transferred to microdrops of 0.1 mL T6 0.4% BSA medium (10 oocytes in each microdrop), covered by light paraffin oil, and incubated until insemination. Sperm cells were added to the microdrops to give a final concentration of 10^5 cells/mL or 10^6 cells/mL as needed.

Fertilization and Embryo Growth

Five hours after insemination, oocytes were collected, washed twice, and transferred to bicarbonate-buffered M16 medium (19) with 0.4% BSA. On the following day, the number of oocytes that had reached the two-cell stage were recorded. These two-cell embryos were further cultured for 4 days, and the rate of blastocyst formation and hatching was determined. Assessment of fertilization and embryo development was performed employing inverted contrast microscopy.

Embryo Transfer (ET) to Pseudopregnant Recipients

Embryos at the blastocyst stage, produced from another pool of similarly laser-drilled oocytes, were transferred, as previously described (19), to the uteri of pseudopregnant white sabra females 3.5 days after coitus with vasectomized males. Embryos originating from laser-drilled and control oocytes from the same pool were transferred to different sabra females. Pregnancies were allowed to proceed to term, and newborns were identified by the pigmentation of their eyes and coats.

Anatomical Examination

Killed animals were examined for gross anatomical defects. The brain, thyroid gland, lungs, liver, spleen, kidneys, adrenals, stomach, duodenum, pancreas, small and large intestine, cecum, ovaries and testicles were placed in Bouin's solution for fixation. Trichrom-stained slides were prepared from these organs and examined by a veterinarian histopathologist.

RESULTS

Generally, the drilled holes were of the same round shape and size as determined by the pipette's tip dimensions, with sharp edges and no signs of damage to the surrounding zona (Fig. 2). In a preliminary

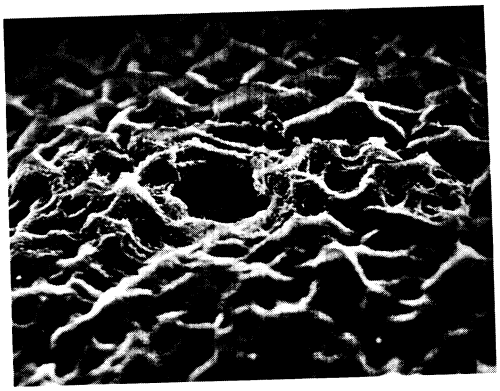

Figure 2 Scanning electron microscopy of a mouse oocyte drilled by a pipette-directed excimer laser beam. Significant features include the sharp circular edges of a 3-μm drilled hole and the well-preserved architecture of the surrounding ZP. (initial magnification was ×10,000).

work we found that the optimal diameter of the pipette tip for this application was 8 micrometers.

Effect of Laser Drilling on Fertilization and Embryo Development In Vitro

Laser drilling was performed on 201 oocytes of which 18 (9%) were damaged: 10 during the preparation and the others when drilling was performed. Evidence for oocytes damage included the disruption of the ooplasm either by vigorous sucking by the holding pipette or direct damage by the treating pipette after ZP. The oocytes were divided into two groups: group I (89 oocytes) was inseminated with 10^5 cells/mL and group II (94 oocytes) was inseminated with 10^6 cells/mL. Both groups' fertilization rate and development in vitro were compared with control oocytes undergoing the same preparation steps but no drilling (94 and 88 oocytes for group I and group II, respectively). Fertilization was significantly ($P < 0.001$) lower for both laser-drilled and control oocytes when 10^5 sperm cells/mL were used for insemination. However, for both sperm concentrations laser drilling significantly ($P < 0.001$) enhanced fertilization over controls. Laser drilling enhanced fertilization from 32% to 67% at 10^5 cell/mL and from 55% to 90% at 10^6 cells/mL (Fig. 3A). The polyspermic fertilization rate was similar for both laser-drilled and control oocyte (1.4% and 0.8%, respectively).

The embryos' morphology was assessed daily up to 96 hours after insemination. At this point, 39% of control and 48% of laser-drilled embryos continued to develop from the two-cell stage to the blastocyst, suggesting that laser drilling did not adversely affect the developmental potential of laser-treated oocytes in vitro (Fig. 3B). In addition, complete hatching at this point was significantly enhanced ($P < 0.001$) by the drilling procedure (Fig. 3C). No trapped embryos were observed up to 120 hours after insemination.

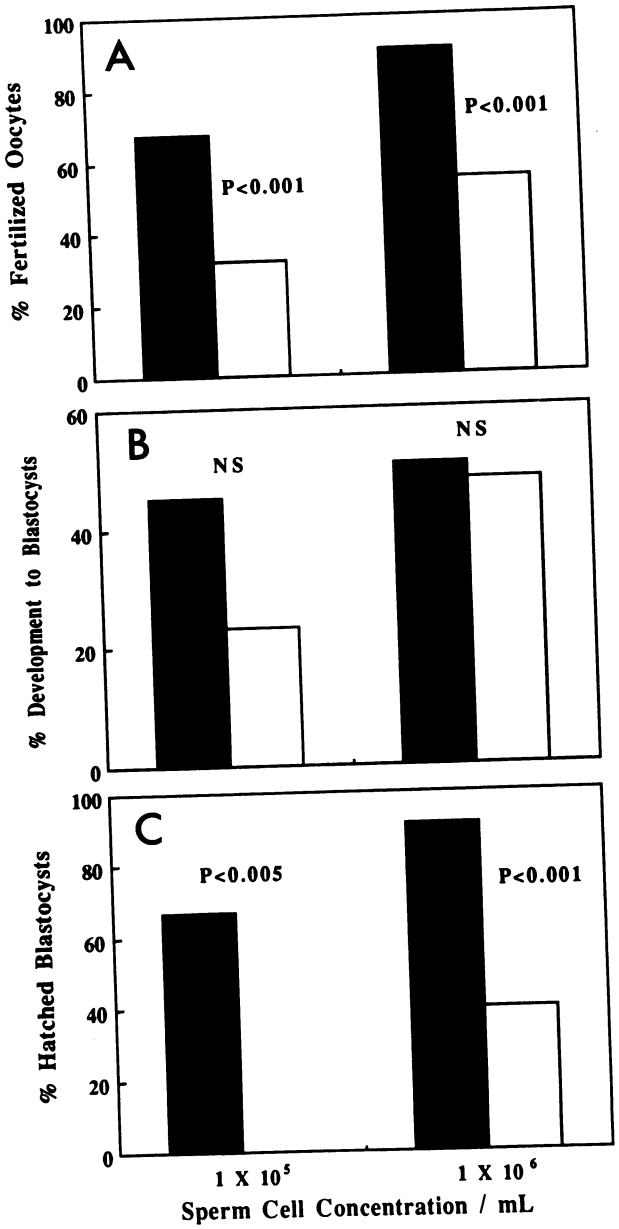

Figure 3 Comparison between the laser-drilled oocytes (■) and control oocytes (□) inseminated with 10^5 cells/mL and 10^6 cells/mL. (**A**), Fertilization rate. (**B**), The development rate from the two-cell stage to the blastocyst stage 96 hours after insemination. (**C**), The rate of blastocysts hatching 96 hours after insemination.

Effect of Laser Drilling on In Vivo Development

One hundred fifty-seven blastocysts developed from laser-drilled oocytes, and 70 blastocysts originating from control oocytes of the same pool were transferred to the uteri of pseudopregnant sabra female mice. Seventeen normal pups, 9 males and 8 females, were born evolving from laser-drilled oocytes (11% efficiency of transfer). One pup was born originating from the control oocytes (1.4% transfer efficiency). All pup males and females were fertile upon maturity when mated with stock CB6F1 opposite sex mice. Two laser-drilled males and 2 females were killed and subjected to a detailed anatomic and histologic examination of internal organs by a veterinarian pathologist. All animals examined were found to be anatomically normal with normal histologic examination of their internal organs, suggesting that laser drilling, at least in this group of animals, was not associated with any untoward developmental effects.

DISCUSSION

The ZP presents a formidable barrier to sperm penetration and contributes significantly to the requirement for a high sperm concentration in routine IVF. This work demonstrates that laser drilling weakened the zona barrier and enhanced sperm in vitro fertilizing ability. At a low concentration (10^5 cells/mL) 8-μm laser-drilled openings increased fertilization to a rate comparable with that achieved with a concentration higher by one order of magnitude.

The most obvious explanation by which zona drilling (chemical, mechanical, or laser) enhances fertilization is that the gap created provides a pathway of direct access to the oocyte. It is, however, possible that creating the hole alters the ZP structure and increases its susceptibility to sperm penetration. Data from experiments employing conditions that preclude normal fertilization such as very low sperm concentration, insemination with acrosome-induced sperm (2), and treatment with anti-ZP glycoprotein 3 antibody (20), have demonstrated that improved IVF for mechanical and chemical drilling is achieved by direct passage via the hole. By employing a model of human sperm penetration into laser-drilled hamster oocytes, we have recently demonstrated that the window created in the ZP was the sole possible path by which human sperm could access the perivitelline space of these rodents' oocytes. It seems therefore that this mechanism of action is common to all three types of drilling. Because of zona elasticity, the opening in the ZP treated by mechanical slitting may seal off. Such reannealing may be impeded by the ablative effect of excimer laser drilling and represents another possible advantage of this technique.

The rate of polyspermic fertilization after laser drilling was low and similar to that of the control group. This rate is comparable with that achieved after zona drilling by acid Tyrode's solution (1) or mechanical cutting of mouse zonae (4) and suggests that from this point of view, laser drilling employing a pipette with a tip of 8 μm is safe.

Our observation, demonstrating that hatching was significantly enhanced by laser drilling, is similar to that of Malter and Cohen (21) employing chemical drilling. Cohen (12) suggested that human embryos resulting from microsurgical fertilization implant to a greater extent than zona-intact embryos. A similar phenomenon of enhanced implantation was also observed after biopsy of eight-cell human embryos (13). Based on a large series of micromanipulated oocytes, three prerequisites for the safe application of routine assisted hatching were defined. First, large holes are more efficient than small holes in supporting hatching. Second, because large holes created before conception may result in loss of blastomeres, this procedure should take place after the third cleavage division. Third, preselection of suitable embryos may be required to achieve good results with this technique (12). A major problem of assisted hatching is the current necessity to use acid Tyrode's solution to create large gaps in the zona. It was suggested that its use is detrimental to both the human oocyte and possibly the preimplantation embryo, especially to blastomeres adjacent to the hole. It may well be that laser drilling with its localized submicrometer ablative properties may be less invasive and replace acid solution in creating openings in the ZP for assisted hatching.

Holes or gaps created in the ZP may affect further embryologic development. Normally, expansion and thinning of the blastocyst's ZP occur before hatching in most mammalian species including the human. However, mouse and human zona that have been micromanipulated do not enlarge or thin. This may consequently cause trapping of the trophoblast and inner cell mass (11, 21). Cohen (12) developed an animal model for assessing abnormalities of hatching after application of micromanipulative techniques. He found that when the size of the hole created mechanically in the zona was narrow (<5 μm),

71% of embryos were trapped. This abnormal hatching could be overcome by drilling larger holes elsewhere in the zona. The absence of trapping in our study suggests that laser-drilled round and well-circumscribed holes of 8 μm allow for normal hatching without interfering with the completion of this process.

The possibility of DNA damage by UV radiation during excimer laser treatment has previously been investigated in a few experimental models. Yeast cells demonstrated a significant amount of DNA repair after excimer irradiation at 193 nm, thus suggesting injury to nucleic acids (22). In contrast, a standard mouse fibroblast cell line exposed to laser irradiation at an identical wavelength did not show an increased anaplastic transformation compared with nonirradiated control cells (23). Similarly, long-term follow-up of corneas after laser keratoplasties demonstrated good healing with no atypical growth (18). It was also demonstrated that this radiation is not mutagenic in a mammalian cell mutagenesis assay (24), and the scattered radiation into tissue during ablation does not initiate unscheduled DNA synthesis (25).

The accuracy of laser zona drilling was previously demonstrated by detailed scanning electron micrographs showing holes with sharp edges and a high degree of precision with minimal damage to the surrounding ZP (16). This work, employing mouse gametes, demonstrates that fertilization and in vitro development to the blastocyst stage were not adversely affected by laser drilling and further substantiates the notion that this treatment is safe and has no cytotoxic effects. Furthermore, the fact that normal fertile offsprings were born after the procedure strongly suggests that the energy level used in these experiments is safe. The attrition rate of 9% of oocytes during the preparatory steps of the actual drilling procedure occurred almost exclusively in the initial learning phase of handling the laser and micromanipulation instrumentation.

We therefore conclude that drilling of mouse oocytes by a pipette-guided argon fluoride excimer laser is a precise and reproducible procedure that technically is simpler and more accurate than mechanical or chemical disruption. It results in enhancement of fertilization and hatching and does not interfere with in vitro or in vivo development and future reproductive capacity.

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