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Interaction between human sperm cells and hamster oocytes after argon fluoride excimer laser drilling of the zona pellucida*

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Objectives: To provide conclusive evidence that sperm cells gain access to the perivitelline space exclusively through a laser-drilled opening. To assess the optimal size of the hole and to evaluate the efficacy of laser drilling in comparison with that of mechanical zona dissection.

Design: An interspecies model of human sperm cell that interacts with a laser-drilled or partially zona-dissected hamster oocytes.

Main Outcome Measures: Penetration rate into the perivitelline space as related to the size of the opening (group A [5 μm], group B [10 μm], and group C [15 μm]) and to the sperm cell concentrations (1×10^6, 5×10^6, and 10×10^6 cells/mL) used for insemination.

Results: For each sperm cell concentration, the penetration rate into the perivitelline space was lowest for group A followed by group C and highest for group B. When penetration was compared for each hole size, it was found that sperm concentration had no effect on the rate of penetration in groups A and C but significantly affected this rate in group B. The highest penetration rate of 73% was observed with a concentration of 10×10^6 cell/mL and declined to 58% and 23% at 5×10^6 cell/mL and 1×10^6 cell/mL, respectively. Oocytes drilled by laser (10-μm hole) demonstrated a significantly higher penetration rate when compared with those treated by partial zona dissection (73% versus 20% and 58% versus 21% for sperm densities of 10×10^6 cells/mL and 5×10^6 cells/mL, respectively).

Conclusion: Human sperm cells gain access into the perivitelline space of hamster oocytes exclusively through a hole drilled by an argon fluoride excimer laser. An opening of 10 μm was found to yield optimal results. Laser drilling of the zona pellucida seems to be superior to that of mechanical slitting in terms of sperm oolema interaction. Fertil Steril 1993;60:159–64

Key Words: Micromanipulation, excimer laser, hamster model, partial zona dissection, zona pellucida drilling

Over the past 5 years microassisted fertilization has emerged as a possible tool in the treatment of infertility associated with subfertile sperm. One of the micromanipulative techniques currently in use is the creation of gaps in the zona pellucida (ZP). This technique is carried out by either chemical agents that dissolve the glycoprotein structure of the zona or by mechanical tearing. In the mouse, it has been shown that the interference with the continuity of the ZP enhanced fertilization at low sperm cell concentrations and did not adversely affect the developmental potential of such embryos to normal litters (1, 2). In this animal model, zona drilling with acid Tyrode's and zona cutting by mechanical force were equally efficient in enhancing fertilization (3). Human oocytes, however, seem to be more vulnerable to chemical zona dissolution, and al-

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though fertilization was improved, the development of these treated oocytes into morphologically normal embryos was impaired (4–7). Because in humans no pregnancy was achieved after chemical zona drilling, this technique was abandoned.

In contrast, mechanical zona tearing, also termed partial zona dissection, yielded better results in human IVF. Several studies have shown that this procedure improved the efficiency of IVF in cases of subfertile sperm (4, 5, 8) and resulted in several normal pregnancies (5, 9–12).

The results of partial zona dissection depend mainly on the degree of abnormal sperm parameters used for fertilization (8, 10–14). However, additional factors may contribute to the success rate of zona slitting: the extent of visible and concealed damage incurred to the oocyte during the procedure and the size of the opening created. The major limitation of mechanical zona slitting is the inability to produce standardized and uniform holes. Simon et al. (8) have shown that the average slit after partial zona dissection is rectangular, 10 to 20 μm in length and 2 to 5 μm in width. Larger or smaller holes can be created depending on the size of the piercing pipette and the degree of the sheering forces induced by the operator through the pipettes. An optimal opening must be large enough to allow the interaction between sperm cells and oolema and small enough not to increase the likelihood of polyspermic fertilization or to result in the extrusion of the oocyte from the zona.

To overcome these disadvantages of mechanical zona slitting, we have developed a unique laser driller that applies an argon fluoride excimer ultraviolet beam at a wavelength of 193 nm (15). Using such a system, it was possible to drill extremely precise holes in the ZP of a mouse oocyte without detectable adjacent damage. Moreover, it was shown that oocytes drilled by such a laser beam were able to undergo fertilization in vitro and develop to the blastocyst stage in a rate comparable with that of control oocytes (15). In this animal model, normal sperm was employed, and the possibility remained that sperm cells might have entered the ooplasm via the ZP and not through the gap.

This work, employing an interspecies model, was undertaken to provide conclusive evidence that sperm cells gain access to the perivitelline space exclusively through a laser-drilled opening. In addition, the optimal size of the hole was assessed, and the efficacy of laser drilling was compared with that of mechanical zona dissection.

MATERIALS AND METHODS

Preparation of Gametes

Golden hamster females were superovulated by an injection of 30 IU of pregnant mare gonadotropin (Intervent International, Boxmeer, The Netherlands) followed 48 hours later by 30 IU of hCG (Chorigon; Teva, Petah Tiqva, Israel). The oocyte-cumulus complexes were released from the ampulae into Ham's F-10 medium (GIBCO, Paisley, Scotland) 18 hours after hCG administration. The cumuli were transferred into Ham's F-10 medium containing 0.1% bovine testicular hyaluronidase type I-S (Sigma Chemical Co., St. Louis, MO) and incubated in an atmosphere of 90% N_2, 5% CO_2, 5% O_2 at 37°C for 5 minutes to remove the cumulus cells. The oocytes were rinsed three times in Ham's F-10 medium before micromanipulation.

Semen from a single fertile donor was used throughout the study. The day before oocyte harvesting, specimens were collected, allowed to liquefy, and prepared by centrifugation through a continuous 80% Percoll gradient, as described previously (16). The pellet was washed twice in Ham's F-10 medium supplemented with 10% human serum and centrifuged at 270 $\times$ g for 10 minutes. The final pellet was overlaid with 1 mL of Ham's F-10 medium supplemented with 10% human serum and incubated (90% N_2, 5% CO_2, 5% O_2 at 37°C) for 18 hours.

Micromanipulation by Laser Drilling and Partial Zona Dissection

Laser drilling of the oocytes was performed employing the system used and described previously (15). Briefly, the drilling system used a model 103 MSG, Lambda Physics (Gottingen, Germany) argon fluoride excimer laser with a 193-nm wavelength. The absorption depth of biological materials at 193 nm is extremely small (<1 μm) (17); therefore, with each pulse of the laser beam a thin layer of material can be removed. The laser beam was directed by a series of mirrors and a long focal (f_L = 1,000 mm) lens through the larger end (0.5 mm) of a glass micropipette. The tip of the micropipette was brought in close contact with the ZP, and three to five pulses with an energy fluency of 30 to 50 mJ/cm^2 were required until the zona was penetrated to form a hole. The size of the hole is determined by the diameter of the pipette tip. The laser beam transmission pipettes were pulled from glass capillaries with a 1-mm OD and 0.58-mm ID

(Sutter Instrument Co., Novato, CA). The micropipettes were pulled to the required tip dimension by a Flaming/Brown Micropipette Puller, model P-80/PC (Sutter Instrument Co.). Micropipettes with tips of 5 μm, 10 μm, and 15 μm were produced and employed for creating holes of corresponding sizes.

Partial zona dissection of the hamster ZP was performed as described previously (18) under a Nikon (Diaphot-TMD, Tokyo, Japan) phase-contrast inverted microscope equipped with a heated stage and two Leitz micromanipulators (Ernst Leitz, Wetzlar, Germany). Holding pipettes, for both laser drilling and mechanical slitting, were made using a microflame burner and the tips were polished by fire on a De Fonbrune microforge (MF-1, TPI, St. Louis, MO). Sharp, closed microneedles were pulled from glass capillary tubing (1-mm OD, 0.5-mm ID) using a vertical pipette puller (Model 720; David Kopf Instruments, Tujunga, CA). The microneedle pierced the ZP from one side to another to create a slit of 10 to 15 μm in length and 3 to 5 μm in width.

To reduce possible damage to the oolema, oocytes subjected either to laser drilling or mechanical slitting were manipulated in pairs using a 0.1-M sucrose solution in Ham's F-10 medium, which resulted in an increased volume of the perivitelline space. After micromanipulation all oocytes were rinsed three times in sucrose-free medium. Manipulated oocytes were transferred into 0.2-mL droplets of Ham's F-10 medium containing 10% human serum overlaid with light paraffin oil (M 3516; Sigma Chemical Co.) and kept in an incubator for 2 to 3 hours until insemination.

Laser-drilled oocytes were divided into three groups of hole diameter: 5 μm, 10 μm, and 15 μm. Each group of oocytes was inseminated to three final motile sperm cell concentrations: 1×10^6, 5×10^6, and 10×10^6 motile cells/mL. The same final concentrations were employed for the partial zona dissected oocytes.

Eight hours after insemination (10 to 11 hours after micromanipulation), the oocytes were washed and observed for the presence of sperm in the perivitelline space by means of phase-contrast microscopy. After the light microscopic assessment, all oocytes were prepared as previously described (19) and observed for sperm penetration into the ooplasm.

Statistical analysis was performed employing χ^2 test and Student's t-test wherever appropriate. Statistical significance was defined as $P < 0.05$.

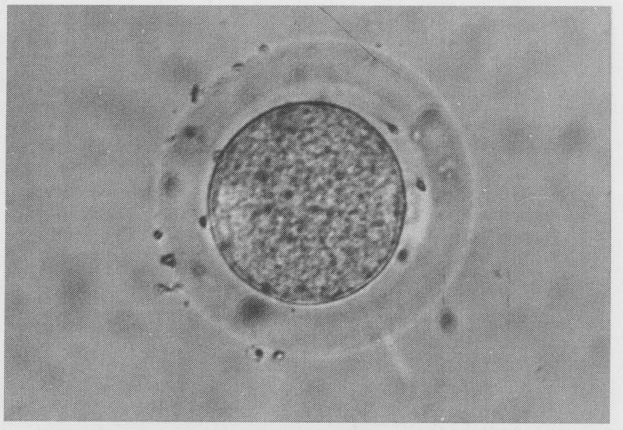

Figure 1 Human sperm cells in the perivitelline space of a hamster oocyte drilled by argon fluoride excimer laser beam.

RESULTS

A total of 315 hamster oocytes were drilled by a laser beam and divided into three groups in which a hole of approximately 5 μm (117 oocytes), 10 μm (110 oocytes), and 15 μm (88 oocytes) was performed in the zona ZP (groups A, B and C, respectively). Six of the oocytes were damaged during the procedure: 2 of them in group B and the others in group C. Oocytes were damaged primarily because of lysis of the oolema (4 oocytes) or extrusion of the oocyte from the disrupted zona (2 oocytes) during the very beginning of the technique application. Each one of the three oocytes' groups was inseminated with three different human sperm cell concentration as indicated in Table 1. Penetration of human sperm cells into the perivitelline space was observed in 99 of 309 laser-drilled hamster oocytes (Fig. 1). Of these, only 3% also showed actual penetration into the ooplasm.

For each sperm cell concentration the penetration rate into the perivitelline space was lowest for group A followed by group C and highest for group B. This trend is best exemplified at the maximal sperm cell concentration (10×10^6 cell/mL) used in these experiments: 8.9%, 73%, and 33.3% for groups A, B, and C respectively (Table 1). When penetration was compared for each hole size, it was found that sperm concentration had no effect on the rate of penetration in groups A and C, but significantly affected this rate in group B. The highest penetration rate of 73% was observed with a concentration of 10×10^6 cell/mL and declined to 58% at 5×10^6 cell/mL and 23% at 1×10^6 cell/mL ($P < 0.001$) (Fig. 2). A phenomenon specific only to the largest holes (group C) was the protrusion of cytoplasm throughout the opening, which probably acted as plug preventing the entrance of sperm cells

Table 1 Rate of Oocytes With Penetration of Sperm Into the Perivitelline Space and Number of Sperm Cells Found in the Perivitelline Space in Different Sizes of Holes and in Relation to Sperm Concentration

	Group A (5-μm hole)		Group B (10-μm hole)		Group C (15-μm hole)		PZD* group (10 to 15-μm slit)	
Sperm density	Penetrated oocytes	Cells in PVS†	Penetrated oocytes	Cells in PVS	Penetrated oocytes	Cells in PVS	Penetrated oocytes	Cells in PVS
1×10^6	3/32 (15.6)	2.6 ± 0.6‡	7/30 (23)	1.3 ± 0.5§	6/28 (21.4)	1.5 ± 0.6	3/20 (15)	1.6 ± 0.6
5×10^6	7/40 (17.5)	2.1 ± 0.9	19/33 (58)	1.6 ± 0.8‖	13/35 (37)	3.3 ± 1.7	5/24 (21)	2.2 ± 1.0
10×10^6	4/45 (8.9)	3.0 ± 0.8	33/45 (73)	2.6 ± 1.4	7/21 (33.3)	3.1 ± 2.5	11/55 (20)	2.8 ± 2.3

* PZD, partial zona dissected.
† PVS, perivitelline space.
‡ Values are means ± SD and values in parentheses are percents.
§ $P < 0.001$ with respect to sperm density of 10×10^6 cells/ml.
‖ $P < 0.01$ with respect to sperm density of 10×10^6 cells/ml.

into the perivitelline space (Fig. 3). This was observed in 25 (30%) of the treated oocytes in this group and is probably related to the size of the opening.

The mean number of cells observed in the perivitelline space correlated best with sperm density in group B (Table 1).

Oocytes drilled by laser (10-μm hole) demonstrated a significantly higher penetration rate when compared with those treated by partial zona dissection (73% versus 20% and 58% versus 21% for sperm density of 10×10^6 cells/mL and 5×10^6 cells/mL, respectively) (Fig. 4). None of the oocytes after partial zona dissection exhibited a protrusion of the ooplasm through the slit.

DISCUSSION

This work employing an interspecies model demonstrates that human sperm gain access into the perivitelline space of hamster oocytes exclusively through a hole drilled by an argon fluoride excimer laser. In an attempt to optimize the size of the laser drilled opening, we found that a 10-μm hole yielded the best results. A smaller opening, that of 5 μm (group A), did not allow sperm cells to traverse freely through it and therefore only a small portion of the inseminated oocytes was penetrated by sperm cells. The fact that sperm penetration through small holes was not affected by concentration indicates that the opening in the ZP is the main factor impeding sperm from getting access to the perivitelline space. In medium-sized holes (group B), a clear correlation was found between sperm density and rate of penetration (Fig. 2). This fact combined with the observation that the number of cells in the perivitelline space was concentration-dependent, strongly suggest that a 10-μm hole size allows free passage of cells across the zona. The seemingly paradoxical observation that

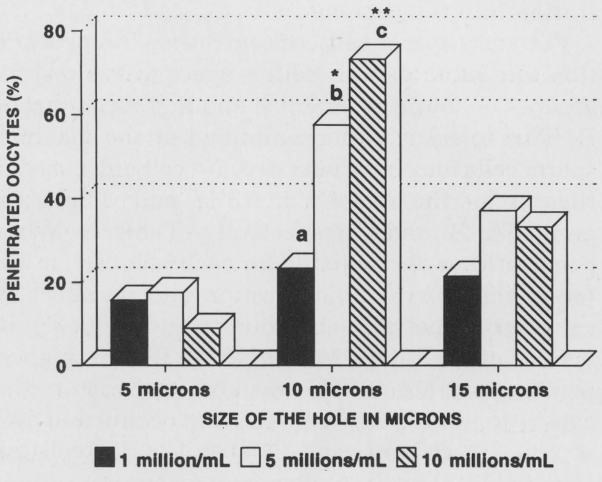

Figure 2 Rate of penetrated oocytes according to the size of the hole in the ZP and in relation to different human sperm cell concentrations used for insemination. *$P = 0.01$ between a and b; **$P < 0.001$ between a and c.

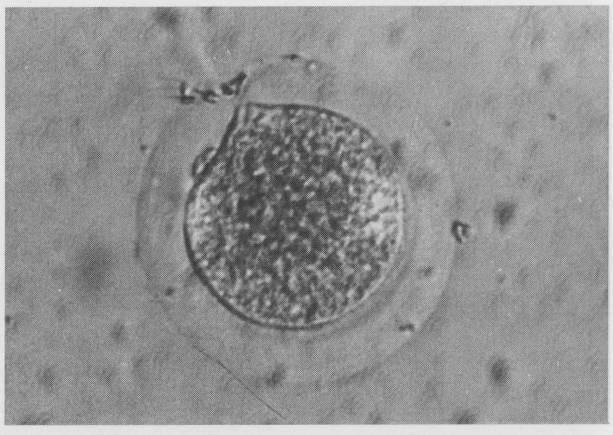

Figure 3 A 15-μm hole in the ZP of a hamster oocyte drilled by an argon fluoride excimer laser beam. The ooplasm plug the opening, impeding the entrance of human sperm cells into the perivitelline space.

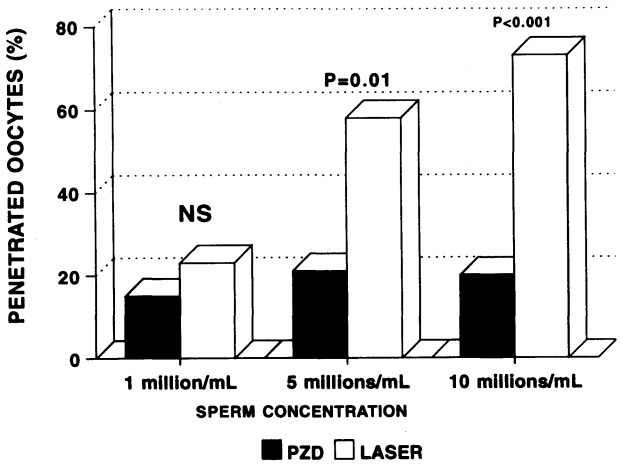

Figure 4 Rate of penetrated oocytes after partial zona dissection and laser drilling (10-μm opening) in relation to human sperm cell concentrations used for insemination.

penetration rate was lower with the largest holes (15 μm) as compared with 10-μm hole, and the fact that sperm concentration did not correlate with penetration suggests that these holes may share physical properties with small-sized holes. Our finding that a large proportion of these oocytes demonstrated protrusion of the cytoplasm through the laser-created gap may explain this unexpected result. It is suggested that in the large-sized holes the hamster ooplasm may plug the opening and impede sperm access into the perivitelline space. Alternatively, in large holes, sperm cells traverse freely in and out of the perivitelline space, thus decreasing the rate of penetrated oocytes.

Creation of holes in the ZP can be accomplished by two other methods: drilling by acid solution and mechanical slitting of the ZP. The advantage of drilling by acid solution is that the holes that are created by dissolution of the glycoprotein structure remain open and allow continuous interaction between the sperm cells and the oocyte. The main drawback of chemical drilling is its deleterious effect on human oocytes (4–7). For this reason it is no longer recommended for use in the human. On the other hand, mechanical partial zona dissection proved to be efficient and successfully applied in human (5, 8, 9). However, it is not yet an ideal method for zona opening. The openings that are created by this method are not uniform in size and shape. This variation in opening sizes affects the success rate of the procedure. Holes that are too large increase the polyspermy rate, whereas narrow slits may close spontaneously and prevent successful interaction between the sperm cells and the oocyte. We present a third method of zona drilling that applies an excimer laser beam. The advantages of the argon fluoride excimer laser beam with a 193-nm wavelength is that when it interacts with the surface of an organic polymer, the material is etched to a submicron depth by photodisruption of molecular bonds without any thermal damage to the biological tissue (15, 17). Moreover, studies applying an excimer laser with an identical wavelength on mammalian cells did not show an increase in anaplastic transformation or unscheduled DNA synthesis (20–22). These advantageous features of the 193-nm excimer laser enables its successful application for human corneal keratoplasties (23) and for cellular microsurgery (15).

The present study demonstrates that laser drilling of a 10-μm opening in the ZP is superior to partial zona dissection in terms of the rate of oocyte penetration by human sperm into the perivitelline space. This advantage is probably due to the ability of the holes created by the laser drilling to remain open. This permits continuous sperm cells exposure to the perivitelline space of oocytes inseminated 2 to 3 hours after drilling. In the case of partial zona dissection, the openings may narrow or even close shortly after the procedure because of the elasticity of the glycoprotein structure of the zona. Thus, if insemination is delayed, it will be difficult for sperm cells to enter the perivitelline space. This possibility is further supported by the fact that sperm concentration had no affect on the penetration rate for the partial zona-dissected oocyte group in contrast to the laser-drilled group (Fig. 4).

The rate of penetration of human sperm cells into the hamster ooplasm was rather low. This low rate reflects the inefficient process of interaction between human sperm cells and a hamster oocyte, as only a totally denuded hamster oocyte that is exposed to many hundreds of thousands of human sperm cells may be penetrated. A few human sperm cells under the ZP of a hamster oocyte may not penetrate the ooplasm. Thus, the sperm penetration assay, as tested in this experiment, cannot assess reliably the viability of the oocyte nor its fertilizing capacity after laser drilling. For such an assessment, an animal model using sperm cells and oocytes from a same species should be employed.

In this interspecies model, the sperm cell concentrations used were high. The lowest penetration rate into the perivitelline space was found in a concentration of 1×10^6 sperm cells/mL. This low penetration rate achieved with a sperm density in a range similar to that used for human IVF may limit

the application of the technique for clinical use. However, the true efficacy of the technique should be tested in a model of the same species undergoing IVF rather than assessing sperm penetration into the perivitelline space of interspecies oocytes. Moreover, it was recently reported that laser drilling of human oocytes for a male factor infertility (24) and of human embryos for assisted hatching (25) were successfully applied. Therefore, it can be suggested that laser drilling may be used to improve fertilization rate in male factor infertility, to assist blastocyst hatching, and to facilitate embryo biopsy for preimplantation diagnosis. However, as with other zona-opening procedures, the efficacy and safety of such a novel technique should be tested on an animal model before its routine application in the human.

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