

Microheater as an alternative to lasers for in-vitro fertilization applications

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ABSTRACT

During the last decade various lasers have been applied to drilling of the micrometer-sized holes in the zona pellucida of oocytes for in-vitro fertilization applications. In this paper we describe an alternative approach to laser instrumentation based on microfabricated device capable of precise drilling of uniform holes in the zona pellucida of oocytes. This device consists of a thin (1 micrometer) film microheater built on the tip of glass capillary with a diameter varying between a few to a few tens of micrometers. Duration of the pulse of heat produced by this microheater determines the spatial confinement of the heat wave in the surrounding liquid medium. We have demonstrated that gradual microdrilling of the zona pellucida can be accomplished using a series of pulses with duration of about 300 μ s when the microheater was held in contact with the zona pellucida. Pulse energy applied to 20 μ m tip was about 4 μ J. In vitro development and hatching of 127 micromanipulated embryos was compared to 103 non-drilled control embryos. The technique was found to be highly efficient in creating round, uniform, well defined holes with a smooth wall surface, matching the size of the heating source. The architecture of the surrounding zona pellucida was unaffected by the drilling, as demonstrated by scanning electron microscopy. Micromanipulated embryos presented no signs of thermal damage under light microscopy. The rate of blastocyst formation and hatching was similar in the micromanipulated and control groups. Following further testing in animal models, this methodology may be used as a cost-effective alternative to laser-based instrumentation in clinical applications such as assisted hatching and embryo biopsy.

Key words: zona pellucida drilling, assisted hatching, cell surgery, mouse embryo

1. INTRODUCTION

The micromanipulation techniques have brought overwhelming clinical advances in human In-Vitro Fertilization (IVF). These techniques are mainly utilized to aid IVF in cases of male subfertility [1,2] to promote the blastocyst hatching process in cases where retarded embryo hatching is considered as a contributor to implantation failure [3,4], and to facilitate polar body or blastomere sampling for preconception or preimplantation genetic analysis.

Initially, chemical dissolution of the zona pellucida (8 μ m thick wall of the oocyte) has been developed based on application of the Tyrode's solution or chymotrypsin [5], and later mechanical partial zona dissection [6] or slitting was introduced [7]. The main drawback of chemical dissolution is its possible toxicity to oocytes and embryos [8]. Release of acid Tyrode's solution into the narrow perivitelline space of the oocyte or embryo may induce cytoplasmic acidification, resulting in cytoplasmic degeneration [9]. Indeed, human oocytes are significantly damaged when acid Tyrode's solution is employed to create a gap in the zona pellucida [10]. Using chymotrypsin instead of acid Tyrode's solution has proven to be less toxic to the oocyte, but it affects the integrity of the zona pellucida and seriously impairs human embryonic development [10].

The main disadvantages of mechanical breaching are its technical complexity and inability to produce standardized and uniform holes. A hole that is too small may result in blastocyst trapping [3]. A slit that is too large may increase the rate of polyspermic fertilization [10], lead to the premature extrusion of the oocyte or cleavage stage embryo from the zona [11], or allow the leakage of blastomeres [3,11].

To overcome these disadvantages, several laser-based techniques have been developed to breach the zona pellucida either in a 'contact mode' or in a 'non-contact mode'. Lasers that function in a 'contact mode' operate at a wavelength that has

a sub-micron penetration depth in tissue or in water, and therefore their energy has to be delivered to the zona through a tapered micropipette or an optical fiber. The zona is dissolved by a photochemical [12] or a photothermal mechanism [13], and the geometry of the opening matches the micropipette tip. These lasers have proved effective, and were successfully used in clinical IVF programs leading to pregnancies and the birth of healthy offspring [9,13,14]. Still, they are costly and their utilization requires a certain level of technical expertise and warrant the manufacturing and reesterilization of pipettes and fibers [15, 16].

To circumvent the 'contact mode' demand of light delivery by a micropipette or a fiber, lasers that operate in a 'non-contact mode' have been applied. In this approach the deep penetrating radiation (near-infrared) is tightly focused through the microscope objective on the periphery of the zona pellucida [17,18]. Two cylindrical holes or a slit are created by photothermolysis of the zona which is placed tangentially to the laser beam. So far this technique has proved effective and safe only in the mouse model [18]. The laser which has been used in these latter studies is a relatively simple diode laser which is very cost-effective. However, at the emission wavelength of this laser, a significant heat deposition is estimated [19] to be conducted through a relatively large distance from the drilling site and this is of concern.

In this paper we describe a simple methodology for zona pellucida breaching that has a potential of eliminating the need for the use of the "contact mode" lasers in this procedure. Specifically, we use a heating microdevice emulating the localized photothermal action of the shallow penetrating laser radiation. Similar to the laser systems, our method allows for creation of the precise, reproducible and uniform holes in the zona pellucida.

2. MICROHEATER VERSUS LASERS - THE CONCEPT

One-dimensional propagation of the heat wave in water from the flat source of heat with constant temperature ΔT_0 above the ambient temperature positioned at $x=0$ can be described as following:

$$\Delta T(x,t) = \Delta T_0 \cdot \operatorname{erfc}\left(\frac{x}{2\sqrt{at}}\right),$$

Where function $\operatorname{erfc}(z) = \frac{2}{\sqrt{\pi}} \int_z^{\infty} e^{-t^2} dt$, and a is thermal conductivity ($1.4 \cdot 10^{-3} \text{ cm}^2/\text{s}$ for water).

Penetration depth of the wave of heat in water can be estimated as following: $h = \sqrt{at}$ (FWHM). Thus duration t of the pulse of heat generated by the source determines the initial depth of heated volume of water adjacent to its surface. Since the $\operatorname{erfc}(x)$ function descends faster than $\exp(-x)$, the microheater is capable of generating the heat wave with better control of penetration depth than any laser. (This will only be true assuming the heat capacity of the source being negligible as compared to that of the heated volume of water.) For example, $t = 7 \mu\text{s}$ will correspond to $h = 1 \mu\text{m}$, and $t = 300 \mu\text{s}$ to $h = 6.5 \mu\text{m}$, respectively. Thus, control of the duration of heat deposition will be equivalent to control of the laser penetration depth. The heat will diffuse from the heating film into water and into the glass supporting the heating film. The power deposition required in order to keep the constant temperature ΔT_0 of the heating film will be:

$$P = \left(\frac{\lambda_1}{\sqrt{a_1}} + \frac{\lambda_2}{\sqrt{a_2}} \right) \frac{\sqrt{\pi} \cdot D \cdot \Delta T_0}{4\sqrt{t}},$$

Where λ is thermal conductivity and D is tip diameter. Indexes 1 and 2 stand for water and glass, respectively.

The total energy deposited during the pulse with duration τ can be estimated as following:

$$E = \left(\frac{\lambda_1}{\sqrt{a_1}} + \frac{\lambda_2}{\sqrt{a_2}} \right) \frac{\sqrt{\pi} \cdot D \cdot \Delta T_0 \sqrt{\tau}}{2}$$

For example, with the tip diameter of 20 μm about 1.2 μJ should be deposited in order to maintain the ΔT_0 of 70 $^\circ\text{C}$ during the pulse of 300 μs . After the heat has been deposited, it will diffuse in the surrounding water and tissue similarly to the heat wave deposited by the shallow penetrating laser radiation.

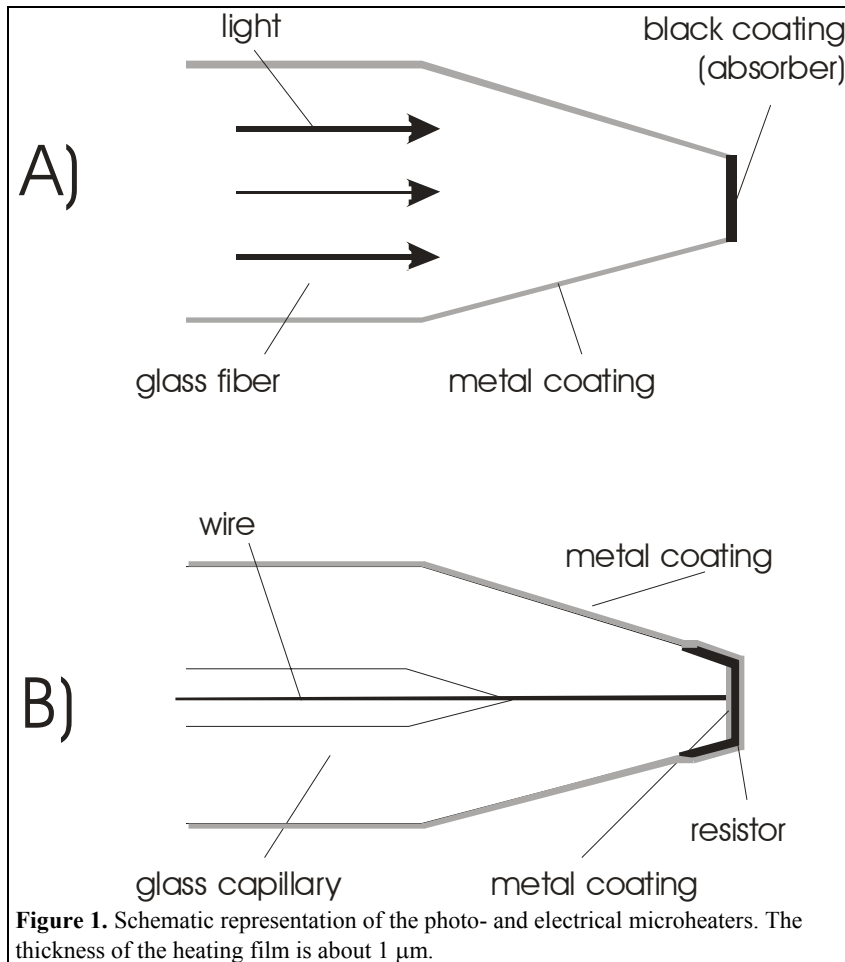


Figure 1. Schematic representation of the photo- and electrical microheaters. The thickness of the heating film is about 1 μm .

Two possible structures of microheaters are shown schematically in Figure 1. The first (A) is a photo-heater, where a thin black film deposited at the exit of the tapered fiber will be heated by the pulse of light. The source of light could be a flash lamp or LED with controlled pulse duration. The thickness of the film should be submicron in order to limit the influence of its own heat capacity on dimensions of the heat wave generated in surrounding water. Another, more convenient version is electrical microheater shown in Figure 1B. The current passing through a thin layer of resistor deposited between the two layers of metal will heat the structure. In order to prevent the leakage of heat through the metal electrodes, the thickness of the internal wire should be much smaller than the tip diameter, and the external metal coating should be submicron in thickness. Although all these requirements do not present major technical problems, we tested this idea using the first version of the microheater, as we did not have an access to sophisticated film deposition equipment.

3. MATERIALS AND METHODS

Experimental setup

Optical fiber with a core diameter of 200 μm and a cladding diameter of 220 μm was tapered using a pipette puller (Model 2000, Sutter Instruments, Inc, Navato, CA, USA). The tip was then polished to form a flat disc with a diameter of 20 μm and coated with a thin (about 1 μm) layer of black dye (see Figure 1A). Residual transmission through the coating was below 1%. To prevent the light leakage through the side walls of the tip, they were coated with a thin (100 nm) layer of Aluminum. 300 μs pulses of light were generated using the Ar⁺ Ion Laser (Model 52 Ion Laser, Coherent Inc., Mountain View, CA, USA) and a home-made shutter. The black coating was heated as it absorbed the light transmitted to the tip and resulted in a gradual microdrilling of the zona pellucida of oocytes held in a gentle contact with the tip.

Embryo collection and culture

Embryos were obtained from CB6-F1 female 6 week old mice. Superovulation was induced by intraperitoneal injection of 5 IU of pregnant mare serum gonadotropin (Sigma Inc., St. Louis, MO) followed 48 hours later by 5 IU of human chorionic gonadotropin (hCG, Sigma). The females were individually housed with stud males of the same species for overnight mating that was verified by the presence of a vaginal coagulation plug the following morning. The females with vaginal plugs were then isolated and further housed. They were sacrificed by cervical dislocation 66 hours post hCG administration, the oviducts removed and dissected in physiological M2 medium (Sigma), and embryos at the 8/16 cell stage of preimplantation development were retrieved. The embryos were pooled, washed twice and randomly placed in groups of 6-10 into 50 μl droplets of M2 medium (Sigma), overlayers with equilibrated paraffin oil (Sigma) in a Petri dish, at 37 $^\circ\text{C}$.

Half of the groups of embryos were randomly assigned to zona pellucida drilling while the other served as controls and remained in their holding drop until the completion of the micromanipulation procedure. The groups of both the control and micromanipulated embryos were then washed and transferred to 50 μ l droplets of M16 medium (Sigma), covered with equilibrated paraffin oil for further culturing at 37°C in 5% CO₂ in air. Observations were periodically made every 20-24 hours over 3 days to assess preimplantation development. The results of the observations of the micromanipulated and control groups were compared by using χ^2 analysis.

Drilling procedure

The micromanipulation apparatus consisted of an inverted microscope (Axiovert 35, Zeiss) fitted with left and right mechanical micromanipulators. Holding pipettes were pulled from capillary tubing using a micropipette and fire polished on a microforge (Technical Products International, St. Louis). The culture dish was placed on the microscope heating stage (37°C) and the 50 \times objective was selected for the drilling procedure. After the embryos were secured to the holding pipette by suction, the tip of the optical fiber was brought into direct contact with the zona pellucida. To avoid damage to the plasma membrane, drilling was performed at sites where the zona pellucida was separated by a few microns from the blastomeres' wall.

Scanning Electron Microscopy

For scanning electron microscopy, the embryos were immediately fixed following the microdrilling in 1% glutaraldehyde for 1 hour. An additional fixation step was carried out in 2% glutaraldehyde solution for 1 hour. Following fixation, embryos were placed on a poly-L-lysine (Sigma Chemical Co.) coated coverslip and fixed with 1% osmium tetroxide for 1 hour. Specimens were then dehydrated through an ethanol series which was followed by a critical point dehydration with CO₂. They were subsequently coated with gold in a Polaron sputter ES-100 coater and examined with a Philips 505 scanning electron microscope.

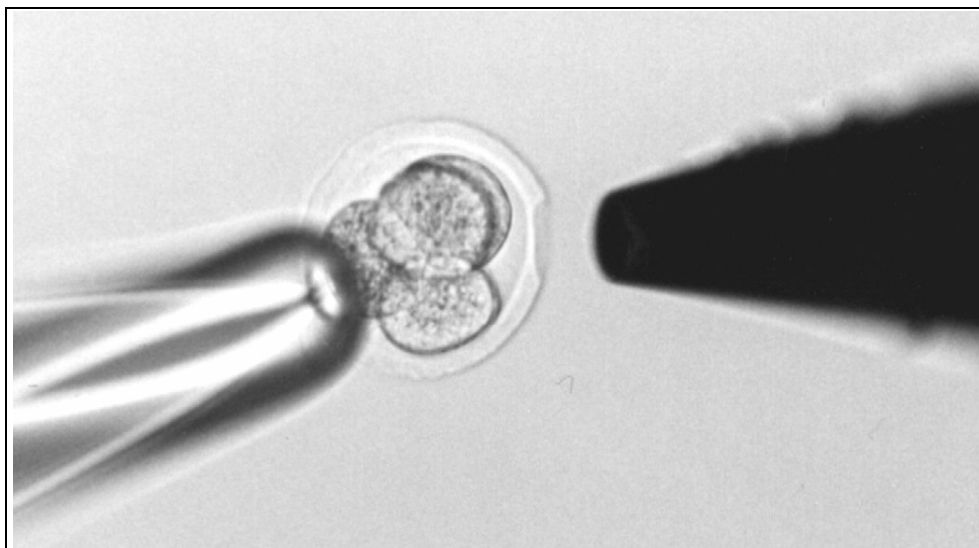


Figure 2. Light micrograph of the cleavage stage mouse embryo during the drilling procedure. After a few pulses, breaching of the zona is still incomplete. The resulting crater has flat bottom and matches in diameter the size of the tip. The tip was withdrawn from contact to allow better observation of the gap.

4.Results

Zona pellucida drilling

Similar to the laser photolysis systems, our method allowed for creation of precise reproducible and uniform holes even with tips as large as 20 μ m. Between 10 to 15 pulses of 4 μ J and 0.3 ms duration were required to create a full-depth gap in the zona pellucida with the tip diameter of 20 μ m. The width of the hole matched the diameter of the tip and it was possible to control the depth of drilling by the number of pulses. A half-depth hole in the zona pellucida with a diameter matching the tip size

(20 μ m) is demonstrated in Figure 2. As can be seen on this Figure, the bottom of the crater is flat indicating that melting of zona pellucida is very well-confined near the tip. Light microscope observations of drilling showed no signs of damage to adjacent blastomeres or gas bubble formation.

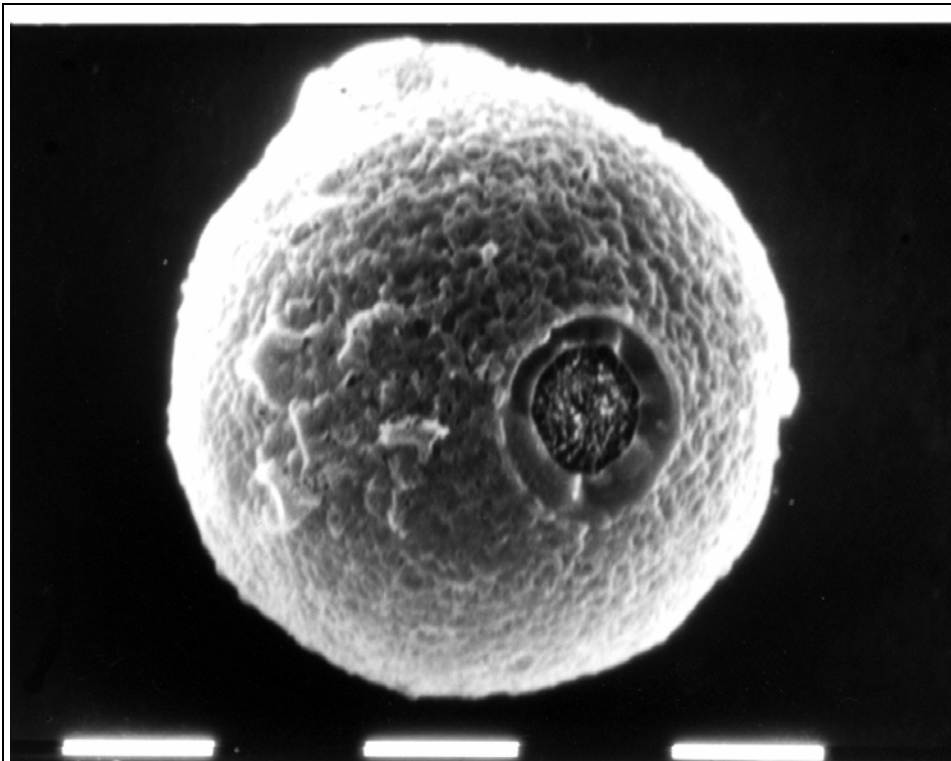
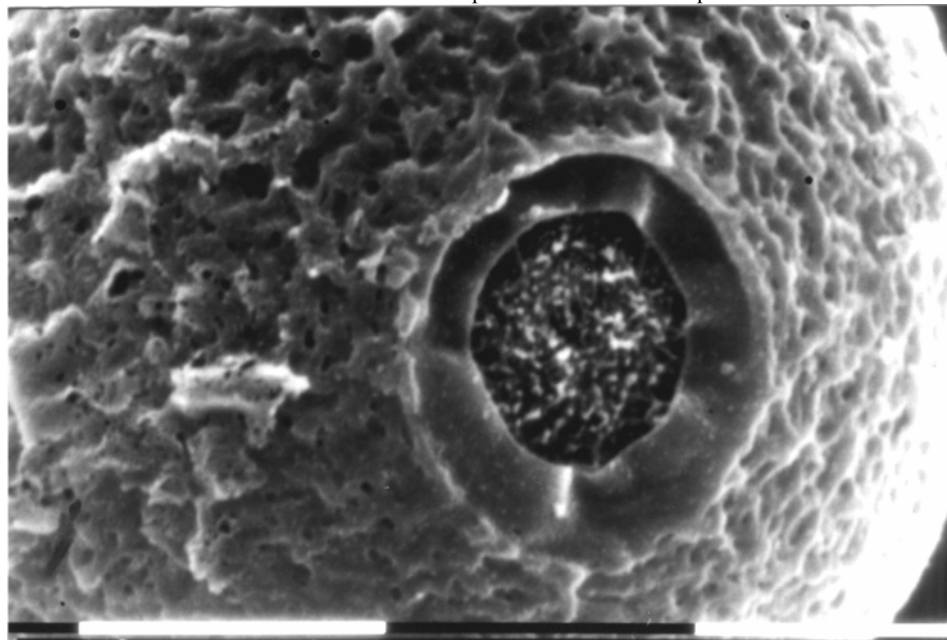


Figure 3: Scanning electron micrograph of the drilled hole in a mouse embryo. **A.** A round well-defined hole with a contour that matches the shape of the microheater tip.



B. The webbed mesh architecture of the zona pellucida around the crater is preserved, while the wall of the crater is smooth indicating on melting.

In order to better characterize the drilled holes and the effect of the heating microdevice on the integrity of the zona pellucida, scanning electron microscopy (SEM) was performed. Figure 4 shows a SEM photograph of a mouse embryo following the zona pellucida microdrilling. A round well circumscribed hole with a smooth wall surface, matching the contour of the optical fiber tip, is demonstrated. The webbed mesh architecture of the surrounding zona pellucida is fully preserved, while the hole wall is smooth indicating on melting of the material during thermolysis.

Effect of drilling on embryo development in vitro

We evaluated the influence of the drilling procedure on the development competence and mode of hatching of mouse embryos. Two hundred and thirty embryos at the 8-16 cell cleavage stage were randomly allocated to two groups. Gaps with a diameter of 20 μ m were created in the zona pellucida of 127 embryos, while the rest 103 embryos were left intact and served as controls.

The rate of blastocyst formation was similar in the micromanipulated and control groups. Forty eight hours after zona drilling, hatching embryos were observed at a significantly higher rate in the study group. The total rate of hatching (partial and complete) did not differ between the groups at 72 hour post zona breaching. However, significantly more embryos in the study group did not complete the process of hatching at that time (see Table

I).

	Zona-intact embryos n=103	Zona-drilled embryos n=127	P*
Blastocyst formation	103 (100%)	123 (96.8%)	0.19
Embryos at 48 hours:			
Hatching	46 (44%)	86 (67%)	0.0008
Hatched	24 (23%)	17 (13%)	0.08
Hatching + hatched	70 (68%)	103 (81%)	0.04
Embryos at 72 hours:			
Hatching	23 (22%)	71 (56%)	< 10 ⁻⁶
Hatched	62 (60%)	40 (33%)	0.00003
Hatching + hatched	85 (82%)	111 (87%)	0.3

Table 1. Development of mouse embryos in vitro following zona drilling at the 8 cell stage.

* χ^2 test. Significance at $p < 0.05$.

5. DISCUSSION

The direct measurement of the temperature field around the microheater during the drilling procedure is rather difficult. It is most probable though that the peak temperature at the tip had to exceed 73°C, which is the threshold temperature for the mouse zona pellucida dissolving [19], but had to be below 100°C as the vapor bubbles formation was not observed (using the microphotography with 1 μ s flash duration). The discrepancy between the theoretical estimation of the pulse energy required for heating of water in front of the tip by 70°C during the 0.3 ms pulse (1.2 μ J), and the pulse energy required for melting of the zona pellucida in our experiment (4 μ J) may result from several reasons. First one is the fact that part of the light transmitted through the tapered fiber to the tip was reflected from the coating. Unfortunately, we could not measure the reflectivity of this coating directly due to its macroscopic inhomogeneity. The second possible reason of this discrepancy is the fact that in our estimations we did not take into account the heat conductivity of the coating itself (it was not known), and the heat distribution across the coating that will influence the ratio of the heat flow towards the glass (backward) vs. the flow into water (forward).

The safety of thermolysis of the zona pellucida by the 1.48 μ m diode laser [18] was extensively studied in the mouse model. Zona drilling of zygotes as well as metaphase II oocytes, which are extremely sensitive to thermal influences, was shown to have no adverse effect on fertilization, in vitro or in vivo development [18,20]. As the heat flow in our case is three-dimensional, the heat wave should dissipate faster with distance from the tip than in the case of cylindrical heat deposition in a non-contact approach, where the heat flow is only radial. Therefore, it seems that thermal damage to the oocyte is even less likely with the microheater as compared to the non-contact approach.

Since the primary object of the study was to develop a simple and cost-effective drilling method that could be used for cleavage stage embryo biopsy or assisted hatching, the experiments were designed around the eight cell stage, at the time when zona drilling for these purposes is performed in the clinical setup. In accord with previous mouse studies [3,6,21], the rate of hatching was not enhanced following zona breaching at this developmental stage as the majority of zona intact mouse embryos hatch after in-vitro culture from this stage.

It was recently shown that hatching efficiency is directly influenced by the initial embryonic stage cultured. The extent of hatching is reduced when in-vitro culture is initiated at the 2-cell stage or earlier and in such circumstances it may be significantly improved by assisted hatching [22]. Alternatively, the use of protein-free media may also significantly decrease the rate of hatching which may also be rescued by zona drilling [22]. These mouse models could be used in future studies to examine whether zona breaching by our method will be effective in improving hatching ability.

The hatching process may be altered in several ways by the presence of an artificial hole in the zona pellucida. It was previously shown that following zona drilling, mouse and human embryos hatch without zona pellucida expansion, and squeeze prematurely through the artificial hole in a characteristic figure-eight shape [3,6,11]. It has been demonstrated that completion of the hatching process depends on the size of the opening in the zona pellucida [3,6]: when the diameter of the created gap was smaller than 20 μ m, the majority (85%) of embryos were trapped and did not complete hatching. The rate of incomplete hatching was reduced to 36% when the diameter of the holes was 20-40 μ m. We have also observed an increased rate of incomplete hatching 72 hours following zona pellucida drilling which was most probably due to the 20 μ m gap diameter that we have created.

In conclusion, this study demonstrates the principle of a new simple, effective and accurate method for zona pellucida microdrilling by local gradual thermolysis induced with a heating microdevice. This method can generate standardized holes, without adversely effecting in-vitro mouse embryo development. The next step would be the construction of electrical microheater that will eliminate the need for the light pulse generation and thus will be even simpler than its optical prototype described in this paper.

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