ANIMAL INVESTIGATIONS

Assisted Hatching in Mouse Embryos Using a Noncontact Ho:YSGG Laser System

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Purpose: *A noncontact holmium:yttrium scandium gallium garnet (Ho:YSGG) laser system has been designed and tested for the micromanipulation of mammalian embryos. The purpose of this preliminary investigation was to determine the effectiveness of this laser for assisted hatching and evaluate its impact on embryo viability. The Ho:YSGG system, utilizing 250-μsec pulses at a wavelength of 2.1 μm and 4 Hz, was used to remove a portion of the zona pellucida (ZP) of two- to four-cell FVB mouse embryos.*

Results: *In the first experiment there was no difference in blastocyst production or hatching rates following laser or conventional assisted hatching (LAH or AH, respectively) in contrast to control embryos cultured in a 5% CO_2 humidified air incubator at 37°C. In the second experiment a blastocyst antihatching culture model was employed and LAH-treated embryos were cultured in a serum-free HTF medium (HTF-o). Blastocyst formation was not influenced by LAH treatment and hatching was increased ($P < 0.01$) from 4 to 60% compared to HTF-o control group.*

Conclusions: *These preliminary data demonstrate the utility and nontoxic properties of the Ho:YSGG laser system for quick and precise ZP drilling.*

KEY WORDS: micromanipulation; laser; zona pellucida; assisted hatching; embryos.

INTRODUCTION

Micromanipulation of human gametes and embryos has developed into clinically proven methods for assisting fertilization and hatching. Advances in laser technologies may offer potentially useful alternatives for manipulating embryos in a micropipette-free, noncontact mode. The use of laser energy to assist fertilization is possible but is unlikely to match the revolutionizing success of the direct intracytoplasmic sperm injection technique. However, the beneficial applications of the laser will undoubtedly be realized as a simple, noninvasive method for manipulating the zona pellucida (ZP).

Several parameters determine the laser effect on the gametes: wavelength (WL), pulse duration, energy per pulse, pulse repetition rate, total time of radiation exposure, beam spot size, and focal plane (1). The water content of the manipulated object and the surrounding environment (i.e., gas or liquid, water, or oil) may also influence the laser effect (2). The accuracy and simplicity with which a laser can be utilized to open the ZP have already been demonstrated in several studies (1,3,4). Li *et al.* (5) studied topical effects of lasing on mouse blastomeres using a 308-nm excimer laser. Effects were monitored by microinjection of a vital fluorescence

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dye into the cell immediately adjacent to the site of zona photoablation. Their data suggested that the 308-nm excimer laser had a detrimental effect on precompacted mouse embryos, however, a different choice of laser parameters could have corrected this problem.

Further analysis on embryonic development was subsequently evaluated by Neev *et al.* (6) using the same excimer laser (308 nm). Zonae of 8- to 16-cell mouse embryos were either lased (n = 189), zona drilled with acidified Tyrode's solution (n = 183), or left zona intact (n = 188). Blastocyst formation (99–100%) was similar in the three groups. Hatching occurred earlier in the lased embryos compared to those of the control groups. Significantly more embryos were hatched on days 4 and 7 in the conventionally drilled group compared to the laser-treated group. However, implantation rates of morphologically normal laser ablated embryos were not impaired when compared to the control embryos.

The Ho:YAG laser operating in the infrared range (2.1 μm) and delivered through a silicon fiber was applied on the ZP of two- to eight-cell-stage mouse embryos to assist hatching (7). More ($P < 0.01$) blastocysts hatched (67%) following laser treatment, compared to 44% for untreated controls. Feichtinger *et al.* (8) applied another infrared laser (Er:YAG, 2.94 μm) to mouse embryos and, later, to human embryos. In mouse experiments there was no difference in embryo development between treatments; however, on day 3, complete hatching was significantly enhanced in the laser treated group (80%) over the control group (29.3%). The same laser was proven effective in a multicenter study for human AH (8).

Another infrared laser operating in the noncontact mode at 1.48 μm was recently delivered through a 40× objective of an inverted microscope (2- to 4-μm spot diameter, 10- to 40-msec pulse, 0.5–1.2 mJ) to produce LZD in mouse zygotes (9). One discharge was sufficient to drill openings in the ZP ranging from 5 to 20 μm, depending on the laser power and exposure time. Seventy percent of drilled zygotes developed to the blastocyst stage, comparable to the control, and there was no evidence of thermal damage. The development and use of noncontact methods of LAH are critical to their potentially routine clinical use.

In our study, we have investigated the use of the normal-mode pulse Ho:YSGG laser for the controlled removal of the zona pellucida. The 2.1-μm wavelength offers a noncontact mode of operation and can effectively remove the zona pellucida without excessive thermal damage. In addition, its radiation is removed from any absorbing peak of DNA molecules and has never been associated with any mutagenetic or toxic effects. The objectives of this preliminary investigation were to test the efficiency of this laser system for LAH and to determine whether normal embryo development post treatment continues.

MATERIALS AND METHODS

Embryo Recovery, Handling, and Culture

Immature FVB/N (albino) and FVB/N-B (black) female mice (3–4 weeks old) were administered (i.p.) 5 IU eCG, followed 48 hr later with 5 IU hCG, and were placed with isogenic males for mating. Donor mice were sacrificed according to IACUC approved guidelines (ARC No. 92-1457), the oviducts excised, and two- to four-cell embryos recovered (46–54 hr post-hCG) in Dulbecco's phosphate-buffered saline (D-PBS; Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal bovine serum (FBS; Gemini Bioproducts Inc., San Diego, CA).

In Experiment 1, four-cell embryos were assigned to one of three treatments: control culture (n = 63), laser zona dissection (LZD; n = 68), or conventional zona drilling (n = 97). The embryos were in vitro cultured in Dulbecco's modified Eagles medium (DMEM; Irvine Scientific) containing 10% FBS, and the extent of embryonic development was monitored.

In Experiment 2, two-cell embryos were exposed either to laser zona drilling or to control culture in a 5% CO_2 humidified-air atmosphere. All embryos were initially cultured in CZB medium without glucose (10) until the four-cell stage (6–18 hr). Laser-treated (n = 50) embryos were then placed into a simple, protein-free medium (i.e., HTF without serum) to inhibit blastocyst hatching (11). Control embryos were assigned to one of two culture medium treatments: of (i) mHTF with 10% FBS (HTF-s; n = 50) or (ii) mHTF only (HTF-o; n = 50).

In both experiments, embryos were cultured in vitro for 72 to 96 hr in 24-well culture plates (Falcon 3046, Fischer Scientific, Tustin, CA) containing 1 ml of medium/well in a 5% CO_2 humidified-air incubator. Embryo development was monitored every 24 hr, with the rate of blastocyst formation and hatch-

ing being the two critical measurements subjected to chi-square analysis.

Micromanipulation

Conventional assisted hatching was performed using a Narshige micromanipulation system attached to a Nikon Diaphot inverted microscope. Embryos were placed into 50 μl of D-PBS medium + 10% FBS and 0.1 *M* sucrose covered with mineral oil on a culture dish lid (Falcon 3001) and maintained at 37°C. During micromanipulation, each embryo was secured in place using a holding pipette (O.D., 100 μm; I.D. at tip, 20 μm) attached to a microsyringe–air system. A hole in the ZP was created using a zona drilling procedure involving the use of acidic Tyrode's (AT) solution. A 10-μm (O.D.) suction pipette containing AT (pH 2.5) was placed adjacent to the ZP, where a hole was made by ejecting a small amount of AT. The erosion of an isolated region of the ZP was carefully monitored. The surrounding medium was aspirated into the suction pipette to reduce any detrimental influence of AT on the blastomeres. Each embryo was removed from the microdroplet and washed four times in 1 ml of DPBS before being returned to in vitro culture in DMEM.

Laser Zona Dissection (LZD)

The basic experimental apparatus (Fig. 1) consisted of a laser beam directed through a mechanical shutter into an input port of an Axiomat inverted microscope (Carl Zeiss, Oberkochen, Germany). The electronic shutter driver/time (Uniblitz Model SD-1000) was set to determine the exposure time duration. An energy meter was moved in and out of the beam's path to measure the pulse energy. Embryos were pipetted into an individual 10-μl microdrops of mHTF under mineral oil on a quartz slide (5 embryos/slide). The slide was then placed on a motorized $x-y$ stage which allowed for accurate positioning of the ZP with respect to the stationary laser beam. The laser beam was generally focused on the ZP adjacent to the pervitelline space, as far as possible from blastomeres. A videocamera and a monitor screen allowed for continuous monitoring and recording throughout the procedure. Subsequently, the efficacy of each procedure was analyzed using a computerized image processor (Imaging Technology Inc. Series 151, Model S7V807F8-841; IBM PC-AT).

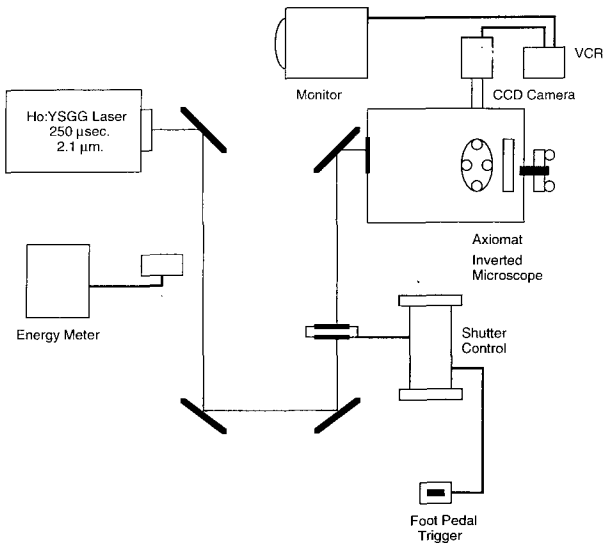

Fig. 1. Experimental Yo:YSSG laser apparatus.

A Ho:YSGG laser (Schwartz Electro-optics, Orlando, FL) at 2.1 μm operating in the normal pulse mode with an overall pulse duration of 250 μsec was employed in our study. Pulse repetition rates were fixed at 4 Hz. This pulse repetition rate was determined in previous experiments to allow pulse-to-pulse thermal relaxation and yet maintain a reasonably fast operation, which is necessary under clinical conditions. Incisions in the zona were obtained by using laser energy (measured at the microscope input) of 7 to 10 mJ per pulse. Since our microscope system transmission efficiency was only about 1%, energy at the target was, therefore, approximately 70 to 100 μJ.

The beam spot size at the target was estimated by observing the size of ablation sites formed by a highly absorbing black ink to the size of 5-μm latex spheres. As with the rest of our experiments, the entire sequence was monitored with a videocamera (Newvicon Dage-MTI series 68, IND) attached to the microscope and recorded on videocassettes. The video images were then transferred to a computer (Macintosh Quadra 840 AV) for further processing. On the computer, image sizes could be calibrated with respect to the known image size of the 5-μm sphere, and spot size as well as incision size could be estimated. While this method is not ideal in that it does not yield precise information on the laser beam profile at the target, it is sufficient for our initial characterization purposes and avoids the need to employ other methods which are of considerably higher technical complexity.

Laser-assisted hatching (LAH) was performed using a spot size of about 10 μm in diameter to create a round tunnel tangent to the ZP inner surface. Two additional holes tangent to and on each side of the original hole were then produced, creating a thinned ZP with an opening of approximately 20 μm. This was done to ensure that the hatching hole is sufficiently large to prevent embryo trapping during the hatching process. The cutting configuration is shown in Fig. 2.

RESULTS

The Ho:YSGG laser allowed for precise ZP removal with well-defined cut boundaries. There were no apparent signs of damage to the surrounding ZP structures or adjacent blastomeres inside (Fig. 3).

There was no difference in blastocyst production or hatching rates among treatments in Experiment 1 (Table I). Laser AH with the Ho:YSGG was rapidly and effectively applied in a noncontact mode, and proved equally effective to conventional AH using acidified Tyrode's solution for promoting early embryo hatching.

In Experiment 2, no difference in blastocyst formation was observed between LAH-treated and control-group embryos (Table I). However, hatching was significantly increased at +96 hr postcollection for LAH-treated embryos compared to HTF-s, which was also higher ($P < 0.001$) than the HTF-o antihatching treatment group.

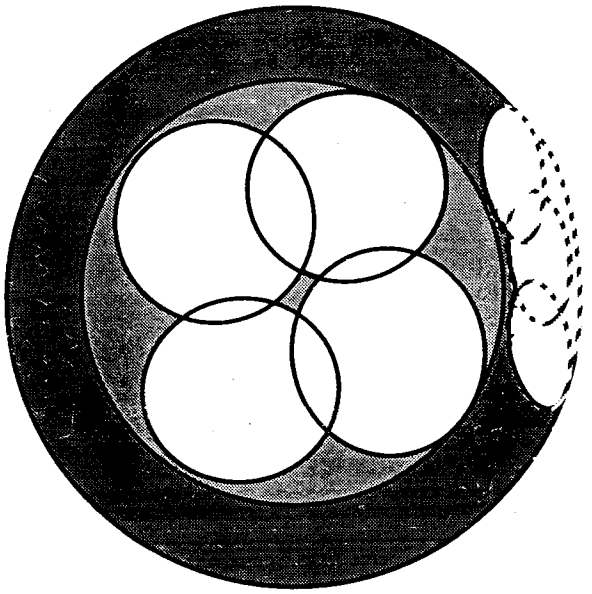

Fig. 2. Cutting configuration for Ho:YSGG laser-assisted hatching.

DISCUSSION

The wavelength region represented by the Ho:YSGG laser emission line may possess the right characteristics of interaction with protein, DNA, water, and glass media (microscope objectives and cover slips) to make it the ideal lasing region for affecting oocytes shapes and for laser micromanipulation. This WL is sufficiently removed from the DNA absorption peak at 260 nm yet still allows efficient interaction with the ZP glycoprotein.

The power of the normal mode Ho:YSGG is high enough that, with IR-coated mirrors, a sufficient amount of energy is transmitted to the target to allow for an effective interaction with the ZP. The 2.1-μm wavelength is absorbed sufficiently well by the proteins to result in a localized interaction even at low pulse energies. At the same time, this wavelength's transmission characteristics through water allows it to propagate through the medium and arrive at the target ZP without prohibitively large energy losses.

To achieve selective ablation, the amount of energy which arrives at the target must be sufficient to cause local vaporization or denaturation of the proteins. Selectivity is achieved by minimizing the amount of heat and other forms of energy which are allowed to propagate to adjacent areas of the cell so that no unwanted damage occurs. Our studies have demonstrated that the Ho:YSGG, with its short pulse configuration, is an ideal candidate for these interaction objectives.

In Experiment 1 we showed that, compared to conventional zona drilling and the nontreated control, the frequency of blastocyst formation and blastocyst hatching was not affected. In Experiment 2 we further demonstrated that LAH acted very effectively to rescue embryos from hatching inhibition, achieving a greater than 19-fold increase in hatching compared to the antihatching control (HTF-o). It appeared that the Ho:YSGG laser could be used effectively and safely on developing embryos.

In previous studies (1,2,5,12) we demonstrated the great utility and precision of the laser (at the appropriate parameters) as a superior device for micromanipulation and precise breaching of the ZP. In

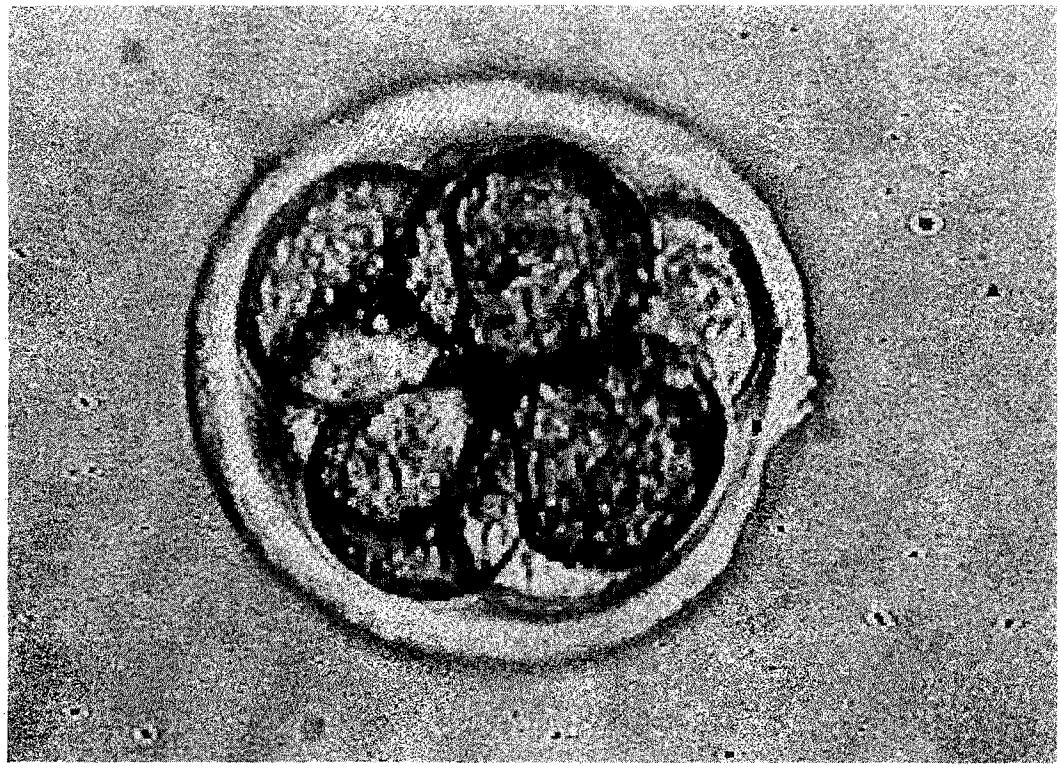

Fig. 3. Microscopic view of Ho:YSGG laser cuts in the mouse zona pelucida.

particular, our results showed the promise of the UV XeCl excimer laser as a tool for such objectives. In this paper we identified a new wavelength regime, this time on the opposite side of the spectrum, which demonstrates the great versatility of the laser but avoided the concerns associated with possible mutagenetic/toxic effects of UV light.

The Ho:YSGG lasers operate in the mid-IR and in a pulse duration (250 μsec) so that peak power is limited to a range where thermal effects are confined, yet mechanical transients which can cause stress-induced damage and tears in the embryos are not generated.

Table I. Blastocyst Formation and Blastocyst Hatching as a Function of Experimental Treatments

Treatment	*n*	No. of blastocysts (%)	No. of blastocysts hatching (%)
Expt 1			
Control	63	62 (99)	49 (78)
AT-AH	97	94 (96)	77 (80)
LAH	68	65 (96)	55 (81)
Expt 2*			
HTF-o	50	46 (92)	2 (4)[a]
HTF-s	50	43 (86)	12 (24)[b]
LAH/HTF-o	50	45 (90)	30 (60)[c]

* Column values with different superscripts in Experiment 2 differ ($P < 0.001$).

The application of UV lasers in a noncontact mode has been proposed by us for a variety of micromanipulation procedures. The noncontact mode has the advantage of operation without the need for disposable fibers, sterilization, and conventional micromanipulation equipment. In modifying the wavelength of the LAH system, we strived still to preserve our noncontact mode of operation. Avoiding the UV absorption peak of deoxyribonucleic acids (DNA) ensures that no potential hazard to the genetic structure can threaten the embryo. Using a short pulse ensures that thermal effects are confined to the desired interaction zone only. The focusing ability of our noncontact objective-based method is limited only by diffraction effects, which means that hole sizes of the order of the laser wavelength can be generated. If beam energy profiles and interaction thresholds are taken into account, the hole size can be even smaller. By adjusting the beam spot size and pulse energy, the interaction time can be minimized to just a few seconds and the entire procedure time to about 1 min. The total energy delivered also can be limited to about three pulses. By

using a single objective in combination with a mobile stage which eliminates the need for expensive micromanipulators, we could potentially reduce the cost of AH micromanipulation procedures. Additional basic studies (including offspring production) to determine any potential hazard of our laser system will soon be concluded in our laboratory.

Our system is simple and quick. It makes use of the basic viewing microscope to achieve simultaneously micromanipulation and AH. It eliminates the need for much of the additional micromanipulation equipment, with the high cost associated with it. As stated above, the animal model demonstrates the lack of embryo toxic effects as well as improved blastocyst hatching. Thus, it is expected that this system may soon be considered safe for human application.

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