Journal of Assisted Reproduction and Genetics, Vol. 15, No. 5, 1998

Successful Use of a Laser for Human Embryo Biopsy in Preimplantation Genetic Diagnosis: Report of Two Cases

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Submitted: December 5, 1997
Accepted: December 8, 1997

Purpose: *The use of Tyrode's acid to drill the zona pellucida for embryo biopsy is the most widely used methodology in preimplantation genetic diagnosis. Instead of this, we propose the use of a 1.48-μm diode noncontact laser, which is quicker, simpler, and safer.*
Methods: *The laser beam was tangentially guided to the zona pellucida of the embryo. Depending on zona pellucida measurement, two to four consecutive shots of 8–22 msec were necessary to drill the zona pellucida of the 13 embryos biopsied for two patients (hemophilia carriers).*
Results: *Female embryos were replaced into the uterus of the patients (1.5 embryos/replacement). One single pregnancy was established (33.3% implantation rate). Coculture of untransferable embryos showed a blastocyst rate of 66.7% (4/6) for male embryos and 25% (1/4) for abnormal ones.*
Conclusions: *These results demonstrate the safety and usefulness of laser methodology in preimplantation genetic diagnosis.*

KEY WORDS: diode laser beam; embryo biopsy; microdrilling; preimplantation diagnosis; zona pellucida.

INTRODUCTION

In 1965, Edwards (1) considered the possibility of introducing the diagnosis of inherited defects in the preimplantation stages of embryos. Nowadays, preimplantation genetic diagnosis (PGD) represents an alternative to prenatal diagnosis and therapeutic abortion in patients with sex-linked diseases (2), single-gene defects (3,4), or aneuploidies (5).

In 1990, Hardy *et al.* (6) reported that the removal of one or two cells from an eight-cell embryo did not have an adverse effect on human embryo development. Handyside *et al.* (2) published the first pregnancies after PGD techniques by Y-specific DNA amplification for sex-linked diseases.

Up to now, new methods have been developed to increase the scope of preimplantation diagnosis of inherited disease and these techniques are now performed at, at least, 14 centers worldwide (7)—a number that is increasing.

Different biopsy methods of mouse and human embryos have been described. Tarin and Handyside (8) classified them in three groups: aspiration, extrusion, and mechanical division. The mechanical division approach requires the removal of the zona pellucida (ZP), which is why this method may be applied to humans only if the zona-free embryos are placed into recipient ZP. In aspiration, blastomeres are removed by suction. The micropipette can be forced through either the ZP or a hole previously made either with Tyrode's acid solution or by mechanical dissection. In extrusion, after drilling the zona, the blastomeres are extruded through the hole by pushing against the zona (9), by displacement of the blastomeres with a gentle flow of medium injected (10,11), or with a microneedle, using stitching movements (8).

The aspiration method is the most widely used for PGD in the human, and it seems that is it not detrimental to embryo development in vitro (6), but it is known that the use of Tyrode's acid solution may reduce the viability of the embryos (12) and their later preimplantation development (13).

In this decade, several laser applications in microsurgical procedures on gametes and embryos have been reported, basically for microsurgical fertiliza-

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1058-0468/98/0500-0302$15.00/0

tion (14,15) or for assisted hatching purposes (16). In all cases, the ZP is microdrilled by exposure to a short laser irradiation that generates standardized holes by local photothermolysis of the protein matrix (17, 18).

There are two types of microbeam laser systems: those that need to maintain contact with the sample (contact laser) (19) and noncontact ones with which the laser microbeam is guided through the optical system of the microscope (noncontact laser) (20). Depending on the wavelength, some emit ultraviolet (UV) radiation (193 nm), which is strongly absorbed by water, present a low penetration depth, and have the potential to cause harmful mutagenic effects (21), while others emit longer wavelengths (266, 308, 355, 366, and 532 nm) and are less absorbed by water. Noncontact lasers have several advantages for clinical applications, but the necessity of finding an appropriate wavelength has been the main problem. Neev *et al.* (22) concluded that the 308-nm XeCl-excimer laser was the most appropriate to induce photoablation of the ZP of mouse embryos, but it was also found to be mutagenic and to increase the incidence of sister chromatid exchange (21).

Feichtinger *et al.* (15) proposed the use of an erbium–yttrium aluminium garnet (YAG) laser that works in the infrared region on a wavelength sufficiently distant from the maximum absorption of DNA to avoid undue impact on the genetic structure of the cells. This type of laser is considered safe, as it allows normal embryo development and healthy live births (23), but it works in the 2.9-μm wavelength, close to the strongest water absorption peak, and as a consequence, the laser light has to be delivered to the target in a contact mode.

Germond *et al.* (24) postulated that the ideal system should provide minimal absorption by the culture dish and the medium, be easily adapted to the microscope, and produce zona drilling with no mechanical, thermal, or mutagenic effects. These authors propose the infrared 1.48-μm wavelength because it fulfills these three criteria. Drilled mouse embryos give rise to normal, fertile offspring. A healthy F_2 generation without neuroanatomical and neurochemical differences was obtained (25).

Laser application in PGD is proposed to improve the efficiency of embryo biopsy by decreasing the time required, avoiding the use of Tyrode's acid solution, and increasing the control of zona drilling.

MATERIALS AND METHODS

Two hemophilia carriers of 34 and 37 years of age underwent an IVF cycle for PGD. Follicular stimulation was carried out by the association of gonadotropin-releasing hormone agonist (GnRHa; Leuprolide; Procrin, Abbot, Madrid, Spain) in a long protocol, with follicle stimulating hormone (FSH-HP; Neofertinorm; Serono, Madrid, Spain) and human chorionic gonadotropin (hCG; Profasi; Serono). The details of this protocol as well as laboratory procedures for IVF have been described previously (26). The culture medium used for IVF was IVF-50 (Scandinavian IVF Science AB, Gothenburg, Sweden). Oocytes were enzymatically denuded of cumulus cells with a solution of hyaluronidase and microinjection was carried out to improve the fertilization rate in metaphase II oocytes. Sixteen (69.6%) of 23 oocytes microinjected were correctly fertilized and kept in culture until day 3. Thirteen embryos cleaved successfuly, whereas three embryos were rejected because of their slow rate of cleavage, the unequal size of the blastomeres, and/or the high percentage of fragmentation. All selected embryos had between four and eight cells at the time of biopsy.

Embryo Biopsy

A 1.48 μm diode noncontact laser (Fertilase; Medical Technologies, Montreux, Switzerland) was adapted to an inverted microscope (Olympus IX 70; Técnicas Médicas MAB, Barcelona, Spain) with two micromanipulators (MM-88 and MO-202 Narishige, Japan), and a 45× objective was used for ZP drilling. The usefulness and safety of this laser system for assisted hatching have already been demonstrated in the mouse and human (17,18,24,25).

This laser was installed in our laboratory to perform assisted hatching as participants in a multicenter study. The study obtained the approval of the ethics committee of our institute.

Each embryo was individually placed in a 50-μl drop of phosphate-buffered solution, Ca^{2+}, Mg^{2+}-free (PBS; Dulbecco's, GIBCO BRL), under mineral oil (Ovoil-150; Scandinavian IVF Science AB, Gothenburg, Sweden), in a plastic dish, at 37°C. The embryo was held with a 35° angle holding pipette (K-HPIP-2135; Cook, Queensland, Australia) and positioned on the aiming spot. The hole had to be drilled close to the blastomere selected for biopsy. It was essential to visualize blastomere nucleus before ZP drilling and blastomere aspiration.

The ZP was measured using a calibrated frame on the video screen to determine the laser exposure time (irradiation time), which is related to the size of the hole that will be obtained. It is important to perforate the ZP completely but not to harm the blastomere with the laser shot. The laser beam was tangentially guided to the embryo to induce a trench in the ZP. Then one blastomere was easily and quickly aspirated with a suction micropipette (K-EBP-3535; Cook).

The biopsied embryos were kept in culture until genetic diagnosis results were obtained and the isolated blastomere was fixed individually according to Tarkowski's method (27), slightly modified (28). Each blastomere was placed in a hypotonic solution (KCl, 75 n*M* in distilled water) containing 10% of synthetic serum substitute (Irvine Scientific, Santa Ana, CA) to prevent attachment of the cell to the dish. After 1 min, the blastomere was transferred to a previously degreased slide, and fixative (methanol:acetic, 3:1) was gently dropped on top of it under stereomicroscope control. The slides were scored under a phase-contrast microscope to locate the nucleus spreads.

The blastomeres were analyzed by FISH as described previously by Munné *et al.* (5). Directly labeled DNA probes for chromosomes X, Y, and 18 were used (Vysis, Naperville, IL). The hybridization targets for chromosomes X and 18 were alpha-satellite repeat clusters in the centromeric region and satellite III DNA at Yqh for chromosome Y. The probes for chromosomes Y and 18 were labeled with red (CEP Spectrum Orange; Vysis) and green (CEP Spectrum Green (Vysis), respectively. For chromosome X a 1:1 mixture of probes labeled with red and green was used (yellow signal).

Embryos diagnosed as normal females for the chromosomes studied (46 XX, 1818) were replaced in the uterine cavity of the patients, on the evening of day 3, using a Wallace catheter (1816 Edwards-Wallace embryo replacement catheter; Simcare, West Sussex, England), while nontransferable embryos (normal male embryos 46 XY, 1818 and abnormal embryos) were kept in coculture in B2 medium (Lab CCD, Paris, France) with a cell support of Vero cells (29) to evaluate the capacity of laser-biopsied embryos to reach the blastocyst stage. Normal male blastocysts were frozen according to the method described by Ménézo and Veiga (30).

RESULTS

A total of 16 cleaved embryos was checked on the morning of day 3, 63–70 hr postinsemination. At that time, seven (43.8%) of them had six or more blastomeres, while the others were at the four- to five-cell stage. A total of 13 embryos (four or more cells) was considered for biopsy.

ZP measurements of the 13 embryos gave us a mean thickness of 18.6 μm (14–24μm). Depending on the ZP of each embryo, 2–4 laser shots ($X = 2.7$) of 8–22 msec ($X = 14.7$ msec) were applied to the ZP to allow zona drilling and subsequent blastomere aspiration (Fig. 1). In a minimum period, a well-defined hole was obtained and embryo biopsy could be quickly and correctly performed (100% biopsy efficiency). Only one blastomere was aspirated from each embryo. A second blastomere was removed in one case because no nuclear material was observed in the spread after fixation (92.9% fixation efficiency).

FISH results were obtained in all embryos biopsied (100% FISH efficiency). Three of the embryos were diagnosed as normal females, six as normal males, and four as abnormal for the chromosomes studies (Table I): two cases of aneuploidies affecting the X chromosome and two alterations of the ploidy. All the embryos diagnosed as abnormal had a slow rate of cleavage (four to five cells at day 3).

Coculture of untransferable embryos showed a blastocyst rate of 66.7% (4/6) for male embryos and 25% (1/4) for the abnormal ones. Spontaneous initiation of hatching was observed in four (80%) of the five blastocysts obtained.

Female embryos were replaced in the uterine cavity (1.5 embryos/transfer) on the evening of day 3. One single clinical pregnancy was evidenced by high levels of plasma β-hCG determination and confirmed by ultrasound examination, actually in the 19th week of amenorrhea.

DISCUSSION

Different laser systems have been used so far to ablate organic material, basically for assisted hatching. Compared to mechanical or chemical disruption of the ZP, the use of a laser is a nontraumatic technique that avoids the distortion of the cell skeleton that may occur in mechanical zona dissection (23); it also eliminates the possible toxicity of the Tyrode's acid solution used in the chemical method.

We chose the 1.48-μm diode noncontact laser (Fertilase) because its safety in assisted hatching had been reported previously both in the mouse and in the human (24,25).

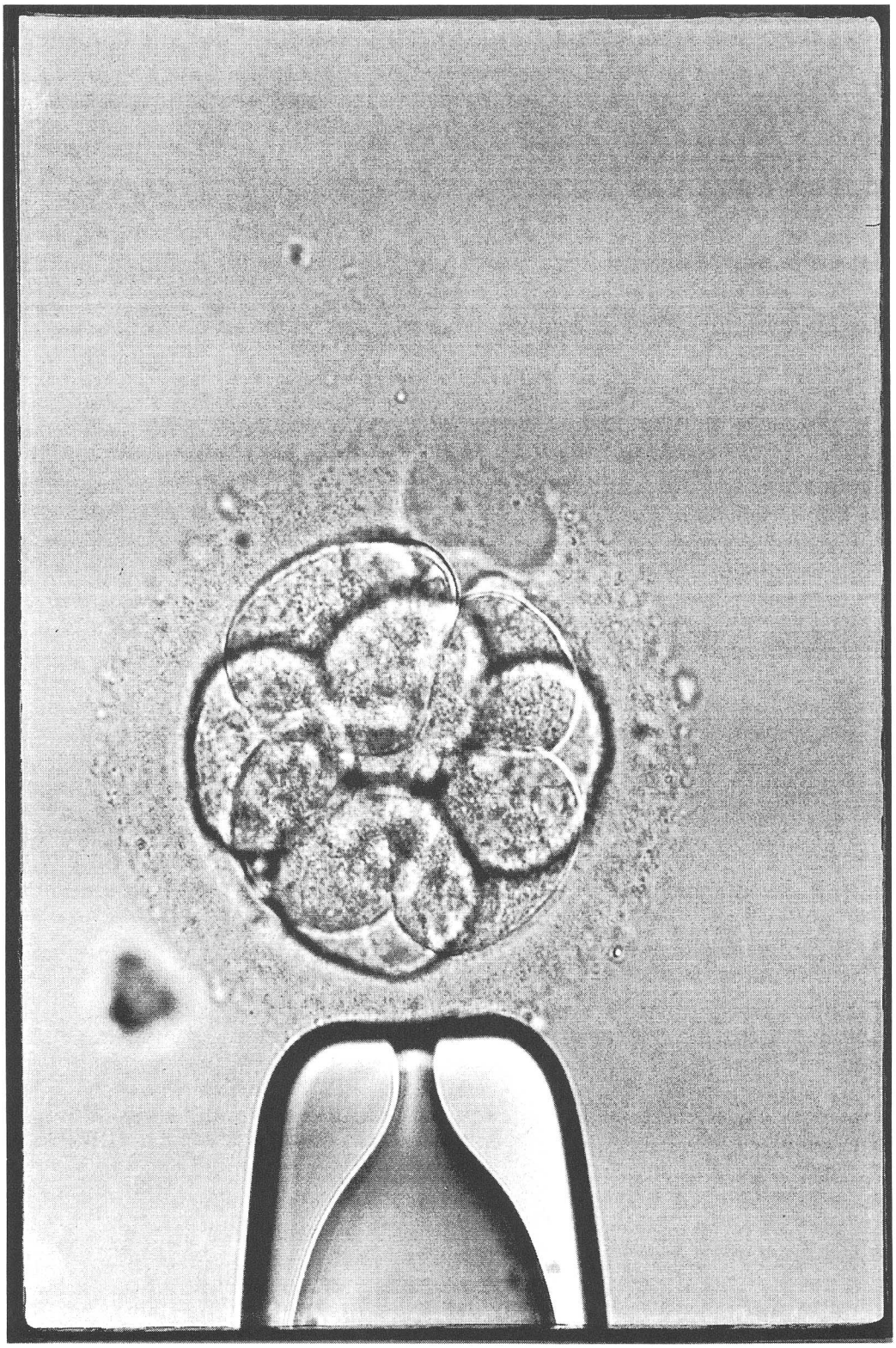

Fig. 1. A well-defined hole obtained after ZP drilling with the use of a 1.48-μm-diode noncontact laser.

Table I. FISH Results of Two Cycles of PGD for Sex Selection with Laser Application for Embryo Biopsy[a]

Patient No.	Embryos biopsied	Blastomeres removed	Female	Male	Abnormal	No diagnosis
1	8	8	2	3	3	0
2	5	6	1	3	1	0
Total	13	14	3	6	4	0

[a] Fixation efficiency, 92%; FISH efficiency, 100%.

Based on our experience, laser application for PGD is proposed because the method is simple, quick, and safe.

The hole in the ZP was made by pressing the laser button and its accuracy produced a well-defined hole exactly in the selected area of the ZP. The ZP was previously measured using a calibrated frame to determine the adequate irradiation time. Blastomere laser exposure has to be avoided. The laser beam was tangentially guided to the embryo to induce a trench in the ZP. An inner irradiation to obtain cylindrical holes as has been suggested for assisted hatching purposes (18) was not necessary. Using this method, the suction pipette was placed exactly in front the hole obtained and no remains of ZP hindered blastomere aspiration. No readjustment of the micropipette was necessary during the procedure. Biopsy could be done quickly and efficiently without changing the micropipette.

The simplicity of this procedure allows its application not only in cleavage embryos but also in metaphase II oocytes of pronuclear-stage zygotes for polar body biopsy (31,32) and on blastocysts for trophoectoderm biopsy (33,34). Therefore, laser application seems to be a useful tool in PGD at different stages.

The ability to reach the blastocyst stage has been used to evaluate the usefulnes and safety of different embryo biopsy methods. In the mouse, Wilton and Trounson (35) and Takenchi *et al.* (36) reported a blastocyst rate after mechanical biopsy of 94 and 83.1–91.5%, respectively. Considering that, in the human, the in vitro blastocyst rate with the use of coculture ranges from 40 to 66% (29), the blastocyst rate observed in these cases and the capacity to hatch spontaneously after laser ZP microdrilling suggest that the procedure described does not negatively affect embryonic development. Furthermore, the pregnancy obtained demonstrates the safety and efficacy of this 1.48-μm diode noncontact laser for PGD.

In conclusion, the blastocyst formation, pregnancy, and implantation rates obtained in these two patients prove that, even though more cases are needed to draw definitive conclusions, laser technology for embryo biopsy in PGD is an efficient alternative to Tyrode's acid. Because of its simplicity, quickness, and safety, the methodology proposed seems to be the actual method of choice for blastomere, trophoectoderm, and polar body biopsy.

ACKNOWLEDGMENTS

The authors thank S. Munné (Institute for Reproductive Medicine and Science, St. Barnabas Medical Center, West Orange, NJ) for technical assistance in FISH methodology, G. Delacrétaz (Institut d'Optique Appliquée, École Polytechnique Fédérale de Lausanne) and K. Rink (Medical Technologies Montreux) for technical support in the use of Fertilase, and M. Germond (Unité de Sterilité, CHUV, Lausanne), scientific director of the multicenter study on laser assisted hatching, through which the laser was incorporated in our laboratory.

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