ANIMAL EXPERIMENTATION

Enhanced Hatching Rate of Bovine IVM/IVF/IVC Blastocysts Using a 1.48- μ m Diode Laser Beam

SEPILL PARK,^{1,4} EUN YOUNG KIM,¹ SAN HYUN YOON,² KIL SAENG CHUNG,³ and JIN HO LIM²

Submitted: July 20, 1998
Accepted: August 27, 1998

Purpose: Our purpose was to test whether zona pellucida (ZP) drilling using a 1.48- μ m diode laser beam on bovine IVM/IVF/IVC blastocysts is effective for embryo hatching.

Methods: Blastocysts produced in vitro at day 7 after IVF were divided into control and laser-drilled groups, respectively.

Results: When the rates of in vitro development of bovine embryos were examined, the average cleavage rate ($\geq$ two-cell) was 82.3% and the blastocyst rate at day 7 after IVF was 32.5%. Using these blastocysts, when the laser drilling effect was investigated at 48 hr after treatment, the total hatching rate in the laser-drilled group (98.0%) was significantly higher than that in the control group (60.0%) ($P < 0.001$). Especially, the hatched rate of the laser-drilled group (68.0%) was significantly enhanced compared with that of the control group (30.0%) ($P < 0.001$).

Conclusions: These results demonstrated that laser ZP drilling on bovine IVM/IVF/IVC blastocysts can significantly increase the hatching rate.

KEY WORDS: bovine IVM/IVF/IVC blastocysts; laser zona pellucida drilling; embryo hatching.

INTRODUCTION

In order to improve fertilization, to facilitate blastocyst hatching, or to perform blastomere biopsy, chemical

(1), mechanical (2) or laser (3) methods have been used to produce holes in the zona pellucida (ZP) of mammalian eggs. Lasers are commonly used in medicine and are especially appreciated for their precise and atraumatic mode of action. Recently, an infrared 1.48- μ m diode laser light, focused through a microscope objective, was shown to allow rapid, easy, and nontouch microdrilling of mouse and human ZP while maintaining a high degree of accuracy under conventional culture conditions (4). Also, the drilling process in the infrared region did not affect embryo survival in mice (5) or in humans (6). Based on these data, this study was performed to examine whether laser ZP drilling on bovine IVM/IVF/IVC blastocysts is effective for embryo hatching.

MATERIALS AND METHODS

Production of Bovine IVM/IVF/IVC Blastocysts

The culture procedures employed in the production of preimplantation embryos from bovine follicular oocytes were similar to those of Rosenkrans *et al.* (7). Briefly, cumulus-oocyte complexes (COCs) were prime collected from visible follicles (2–6 mm) of ovaries, washed with TALP-*N*-(2-hydroxyethyl) piperazine-*N'*-2-ethanesulfonic acid (HEPES) and cultured in maturation medium composed of TCM-199 (GIBCO) + 10% (v/v) fetal bovine serum (FBS) supplemented with sodium pyruvate (0.2 mM), follicle-stimulating hormone (1 μ g/ml), estradiol-17 β (1 μ g/ml), and gentamycin (25 μ g/ml) at 39°C in a 5% CO₂ incubator. After incubation for 22–24 hr in IVM

¹ Maria Infertility Medical Institute, 103-11 Sinseol-Dong Dong-daemun-Gu, Seoul 130-110, Korea.

² Maria Infertility Clinic, Seoul, Korea.

³ Animal Resources Research Center, Kon-Kuk University, Seoul, Korea.

⁴ To whom correspondence should be addressed.

medium, COCs were inseminated using highly motile sperm recovered from frozen-thawed semen separated on a discontinuous Percoll column and heparin (2 μ g/ml) and PHE (18.2 M penicillamine, 9.1 M hypotaurine, and 1.8 M epinephrine) were also added in a fertilization drop for sperm capacitation. At 45 ± 2 hr postinsemination, cleaved embryos ($\geq$ two-cell) were cultured in CR1aa (7) medium. For the study, blastocysts produced in vitro at day 7 after IVF were divided into control and laser-drilled groups, respectively.

ZP Drilling

The setup used for ZP microdrilling has been described in detail by Germond *et al.* (5). Briefly, a 670-nm diode laser aiming beam and the collimated 1.48- μ m cw laser beam (InGaAsP diode laser; MTM Medical Technologies Montreux, Lausanne, Switzerland; purchased from SeYoung Trading Co., Korea) were fed into an inverted microscope through several mirrors and focused by the microscope objective ($\times 45$) within the microscope field in a spot 8 μ m in diameter. The power routinely available at the image plane of the objective was 47 mW, corresponding to a maximal power density of 94 kW/cm².

The classified blastocysts were suspended in groups of 5–10 in a 50- μ l drop of culture dish containing HEPES-buffered medium covered with mineral oil. Using the X–Y microscope stage, each embryo was positioned to bring a region of the ZP on the aiming spot and the ZP was exposed to 3–5 msec of laser light. After treatment (Fig. 1), the embryos were further cultured for 2 days in a cumulus cell monolayered drop containing CR1aa medium supplemented with FBS (10%) and were observed daily for their development morphology to hatching and hatched blastocyst. The control-group embryo was cultured under the same conditions as the treated group.

Statistical Analysis

Differences in the developmental rates between treatment and control groups were assessed using the chi-square test.

RESULTS

The rate of in vitro development of bovine embryos cultured in CR1aa medium after in vitro fertilization was as follows; as shown in Table I, the average cleavage

rate ($\geq$ two-cell) was 82.3% and the blastocyst rate at day 7 after IVF was 32.5%. Also, development rates of classified blastocysts to the early, expanded, and hatching stages were 41.1%, 39.2%, and 9.7%, respectively, when the results were counted from total blastocyst. The results of hatching and hatched rates of bovine IVM/IVF/IVC blastocyst by laser drilling are presented in Table II. When the laser drilling effect was investigated at 48 hr after treatment, the total hatching rate in the laser-drilled group (98.0%) was significantly higher than that in the control group (60.0%) ($P < 0.001$). Especially, the hatched rate of the laser-drilled group (68.0%) was significantly enhanced compared to that of the control group (30.0%) ($P < 0.001$).

DISCUSSION

Artificial opening of the ZP has been proposed to improve the hatching of blastocysts. Two methods of ZP manipulation are already in use to enhance the ability of embryos to hatch: mechanical partial ZP dissection and chemical ZP drilling by acidic Tyrode's solution. Recently, lasers have been commonly used in medicine because of their precise and atraumatic mode of action. There are many types of laser systems used for gamete and embryo micromanipulation such as the 193-nm ArF-excimer laser (8), 308-nm XeCl-excimer laser (9), and 2.9- μ m Er:YAG laser (10).

More recently, efficient and well-controlled laser drilling of the ZP using a 1.48- μ m diode laser was introduced (4). The drilling effect was shown to be due to a highly localized heat-dependent disruption of the ZP glycoprotein matrix, a phenomenon markedly different from the photo ablation induced by wavelengths close to the ultraviolet region (11).

In this study, irradiation conditions were chosen such that the holes could be drilled in the shortest time with a potentially minimal thermal effect considering the thinned ZP of blastocysts (pulse length ≤ 5 ms). As shown in Table II, there are not any degenerated embryos after manipulation by the diode laser.

Generally, expansion and thinning of the blastocyst ZP occur prior to hatching in most mammalian species. In species in which expansion occurs before lysis and implantation, such as the rabbit, bovine, mouse, and human, the ZP becomes extremely attenuated and almost-invisible. However, in the case of artificial micromanipulation, natural expansion of the blastocysts with thinning of the ZP does not take place (2). Thus, the manipulated ZP remains relatively thick (Fig. 2D). Also, an earlier escape of the blastocyst from the

Fig. 1. Bovine embryos drilled with a 1.48- μ m diode laser. (A) Holes of different sizes are drilled by increasing the irradiation time from 3 to 10 msec. In this study, 3–5 msec, was applied to day 7 blastocysts. (B) Drilled expanded blastocyst at day 7 after IVF (arrow). Original magnification, $\times 300$.

ZP environment (4) and an eight-shaped intermediate stage also were observed under our in vitro conditions (Fig. 2C), as indicated in another study (12). However, this study reports that the hatched rate of the laser-drilled group at day 9 (48 hr after treatment) was

68.0%, while that of the control group was 30.0%. These results suggest that laser ZP drilling at the bovine blastocyst stage may be a strong tool to hatch embryos.

Consequently, these results suggested that ZP drilling with a 1.48- μ m diode laser can be used to enhance

Table I. Fertilization and Development Rates of Bovine IVM/IVF/IVC Embryos ($r = 2$)

No. oocytes	No. (%) ≥2-cell	Developed (%) to blastocysts at day 7			
		Total	Early	Expanded	≥ Hatching
463	381 (82.3)	124 (32.5)	51 (41.1)	61 (49.2)	12 (9.7)

Table II. Effect of Laser Drilling on the Hatching of Bovine IVM/IVF/IVC Blastocysts ($r = 2$)

Treatment	Day 7 blastocysts		Developed (%) 48 hr later to*		
	Embryo stage (n)	Total	Hatching	Hatched	Total
Control	Early (25)	50	15 (30.0)	15 (30.0) ^a	30 (60.0) ^a
	Expanded (25)				
Laser drilling ^a	Early (25)	50	15 (30.0)	34 (68.0) ^b	49 (98.0) ^b
	Expanded (25)				

^a Irradiation times, 3.0–5.0 msec.* Means in a column without common superscripts are significantly different ($P < 0.001$).

Fig. 2. In vitro development of drilled and nondrilled (control) bovine blastocysts. Control blastocyst development (A, B) and drilled blastocyst development (C, D). (A) Control blastocysts at day 9 after IVF. (B) Control hatched blastocyst. The ZP has thinned. (C) Drilled blastocyst 48 hr after treatment. The arrow indicates characteristic eight-shaped stage during the hatching process following zona drilling. (D) Drilled hatched blastocyst with a thick ZP. Original magnifications: (A) $\times 75$; (B, D) $\times 100$; (C) $\times 50$ (A, B, C, and D reduced at 93% for reproduction).

the hatching ability of bovine IVM/IVF/IVC blastocysts.

REFERENCES

1. Tucker MJ, Luecke NM, Wiker SR, Wright G: Chemical removal of the outside of the zona pellucida of day 3 human embryos has no impact on implantation rate. *J Assist Reprod Genet* 1993;10:187–191
2. Malter HE, Cohen J: Partial zona dissection of the human oocyte: A non-traumatic method using micromanipulation to assist zona pellucida penetration. *Fertil Steril* 1989;51:139–145
3. Feichtinger W, Strohmer H, Fuhrberg P, Radivojevic K, Antinori S, Pepe G, Versaci C: Photoablation of oocyte zona pellucida by erbium-yag laser for in-vitro fertilisation in severe male infertility [Letter]. *Lancet* 1992;339:811
4. Rink K, Delacretaz C, Salathe RP, Senn A, Norcera D, Germond M: 1.48 μm diode laser microdissection of the zona pellucida of mouse zygotes. *Soc Photo-opt Instrument Eng Proc Ser* 1994;2134A:412–422
5. Germond M, Nocera D, Senn A, Rink K, Delacretaz G, Faken S: Microdissection of mouse and human zona pellucida using a 1.48 μm diode laser beam: efficacy and safety of the procedure. *Fertil Steril* 1995;25:604–611
6. Antinori S, Versaci C, Fuhrberg P, Panci C, Caffa B, Gholami GH: Seventeen live births after the use of an Er:Yag laser in treatment of male factor infertility. *Hum Reprod* 1994;9:1891–1896
7. Rosenkrans CF JR, Zeng GQ, McNamara GT, Schoff PK, First NL: Development of bovine embryos in vitro as affected by energy substrates. *Biol Reprod* 1993;49:459–462
8. Palanker D, Ohad S, Lewis A, Simon A, Shenkar J, Penchas S, Laufer N: Technique for cellular microsurgery using the 193 nm eximer laser. *Lasers Surg Med* 1991;11: 580–586
9. El Danasouri I, Westphal LM, Neev Y, Gebhardt J, Louie D, Berns MW: Zona opening with 308 nm Xe Cl eximer laser improves fertilization by spermatozoa from long-term vasectomized mice. *Hum Reprod* 1993;8:464–466
10. Strohmer H, Feichtinger W: Successful clinical application of laser for micromanipulation in an in vitro fertilization program. *Fertil Steril* 1992;58:212–214
11. Neeve J, Tadir Y, Ho P, Berns MW, Asch RH, Ord T: Microscope-delivered ultraviolet laser zona dissection: principles and practices. *J Assist Reprod Genet* 1992;9:513–523
12. Cohen J, Feldberg D: Effects of the size and number of zona pellucida openings on hatching and trophoblast outgrowth in the mouse embryo. *Mol Reprod Dev* 1991;30:70–78