Preimplantation Development of the Cleavage Stage Embryos after Laser Assisted Embryo Biopsy

Phakphum Phophong, M.D., M.Sc.*
Alpesh Doshi, M.Sc.**
Joyce C Harper, Ph.D.***

Abstract : This study was carried out to determine the effect of duration of exposure to the infrared 1.48 µm diode laser, on the developmental potential of cleavage stage embryos. A total of 69 mouse embryos were included in the study, 22 of which (group A) were biopsied using the laser with a longer duration of exposure (600 ms), while 47 (group B) were biopsied using the same laser with a shorter period (5 ms). The blastocyst formation rate of group B (46/47, 97.8%) was significantly higher than that of group A (12/22, 54.4%). There were no grade 1 blastocysts or hatching in group A. In contrast, 35 of 46 (76.0%) blastocysts in group B were grade 1 and the hatching rate of group B was 84.7% (39/46). In conclusion, the infrared 1.48 µm diode laser may be effective and safe with cautious application. A long duration of exposure to the laser can adversely affect the developmental potential of the biopsied embryos. The laser system with a shorter duration of exposure, therefore, is recommended for laser assisted embryo biopsy.

เรื่องย่อ : การเจริญเติบโตของตัวอ่อนในระยะก่อนการฝังตัวภายหลังการแยกเซลล์บลาสโตเมียร์โดยใช้รังสีเลเซอร์

ภาคภูมิ โพธิ์พงษ์ พ.บ., วท.ม.*, Alpesh Doshi, M.Sc., Joyce C Harper, Ph.D.*****
**ภาควิชาสูติศาสตร์-นรีเวชวิทยา, คณะแพทยศาสตร์ศิริราชพยาบาล, มหาวิทยาลัยมหิดล, กรุงเทพมหานคร 10700, **The Assisted Conception Unit, University College Hospital, UK, ***Department of Obstetrics and Gynaecology, University College London, UK.*

สารศิริราช 2544; 53: 200-206.

ได้ศึกษาในตัวอ่อนหนูจำนวน 69 ตัว เพื่อให้ทราบถึงผลกระทบของความยาวของช่วงเวลาที่ถูกสัมผัสด้วยรังสีเลเซอร์ชนิดอินฟราเรด ขนาดความยาวคลื่น 1.48 ไมครอน ที่มีต่อการเจริญเติบโตของตัวอ่อนภายหลังการแยกเซลล์บลาสโตเมียร์จากตัวอ่อนเพื่อการวินิจฉัยทางพันธุกรรมในระยะก่อนฝังตัว ตัวอ่อนหนูจำนวน 22 ตัวถูกแยกเซลล์บลาสโตเมียร์ออกโดยใช้เลเซอร์ชนิดอินฟราเรดขนาดความยาวคลื่น 1.48 ไมครอน สัมผัสนาน 0.6

**Department of Obstetrics and Gynaecology, Faculty of Medicine Siriraj Hospital, Bangkok, Thailand 10700. **The Assisted Conception Unit, University College Hospital, UK, ***Department of Obstetrics and Gynaecology, University College London, UK.*

วินาที (กลุ่ม 1) ในขณะที่ตัวอ่อนหนูอีก 47 ตัวถูกแยกโดยใช้เลเซอร์ชนิดเดิม สัมผัสนาน 0.005 วินาที (กลุ่ม 2) พบว่าการเจริญเติบโตของตัวอ่อนในกลุ่ม 1 ช้ากว่ากลุ่ม 2 อัตราการเจริญเป็นบลาสโตซิสต์ของกลุ่ม 2 (46/47, ร้อยละ 97.8) สูงกว่ากลุ่ม 1 (12/22, ร้อยละ 54.5) อย่างมีนัยสำคัญทางสถิติ บลาสโตซิสต์ในกลุ่ม 1 ไม่มีลักษณะที่ดีเป็นเกรด 1 และไม่มีการเจริญถึงระดับที่มีเซลล์งอกยื่นออกจากตัวอ่อน ในขณะที่ตัวอ่อนในกลุ่ม 2 ร้อยละ 76 (35/46) มีลักษณะดีเป็นเกรด 1 และสามารถเจริญถึงระดับที่มีเซลล์งอกยื่นออกจากตัวอ่อนได้ถึงร้อยละ 84.7 (39/46) การใช้รังสีเลเซอร์ชนิดอินฟราเรด ขนาดความยาวคลื่น 1.48 ไมครอน มีประสิทธิภาพและปลอดภัยหากนำมาใช้อย่างระมัดระวัง ระยะเวลาที่ตัวอ่อนถูกสัมผัสด้วยเลเซอร์ที่นานเกินไปมีผลเสียต่อการเจริญเติบโตของตัวอ่อน ในการแยกเซลล์บลาสโตเมียร์จากตัวอ่อนเพื่อการวินิจฉัยทางพันธุกรรมในระยะก่อนฝังตัวจึงควรใช้ระยะเวลาที่เลเซอร์สัมผัสกับตัวอ่อนที่สั้น

INTRODUCTION

Preimplantation genetic diagnosis (PGD) has provided the opportunity of detecting single gene defects and aneuploidy as well as structural chromosomal abnormalities in embryos derived from in-vitro fertilisation.[1-3] This technique allows the selection and transfer of unaffected embryos to the couples at risk of inherited disease, resulting in unaffected pregnancies and avoiding of termination of affected pregnancies.[4]

Isolation of genetic material from preconception oocytes or embryos at various stages of preimplantation development is necessary for PGD. Genetic analysis requires one or more cells from the embryos for the diagnosis. Cleavage stage embryo biopsy has been the preferred technique for PGD.[5] Although acid Tyrode's solution has been accepted for drilling an opening in the zona pellucida of the embryos in embryo biopsy, this substance may adversely affect preimplantation development.[6] Laser assisted opening of the zona pellucida by a non-contact infrared 1.48 μm diode laser has been introduced.[7-9] The infrared 1.48 μm diode laser has been reported to be safe both in vitro and in vivo.[7-9] However, more information concerning the safety and use of this laser for embryo biopsy is required, prior to accepting this technique as a standard one.

The purpose of this study was to determine the effect of different exposure periods to the infrared 1.48 μm diode laser on preimplantation development of the embryo from cleavage stage embryo biopsy.

MATERIALS AND METHODS

Cryopreserved cleavage stage mouse embryos strain 3HI, a hybrid of C3H/HCH and 101/H, (Medical Research Council, Oxford) were used in this study. The cryopreserved mouse embryos were thawed and incubated under culture condition of 37°C and 6 % carbon dioxide for 1 hour. Only em-bryos containing 6 blastomeres or more were enrolled in the study. The embryos were divided into two groups with regard to the two different exposure periods of the infrared 1.48 μm diode laser which were used for drilling the zona pellucida of the embryos. The embryos were biopsied, using a laser assisted micromanipulator (Research Instruments Limited, UK). The set-up for zona pellucida drilling was performed according to Germond et al.[8,10] The power routinely available at the image plane of the objective was 1,480 nm/110 mW, pulse mode was up to 750 ms, corresponding to a maximal power density of 380 kW/cm^2. The power output of the laser was constant at all settings, the size of the drilled opening being determined by the duration of the pulse of the laser. Group A embryos were exposed to the laser for 600 ms. The exposure period of group B was 5 ms. Pictures of the biopsy procedure are shown in Figure 1. The embryo was immobilised with a holding pipette and the laser was aimed at a position on the zona pellucida at 3 o'clock. The zona pellucida was exposed to the laser beam with a power that gradually dissolved the zona pellucida from the outer surface until perforation of the zona pellucida occurred. It was important to perforate the zona pellucida

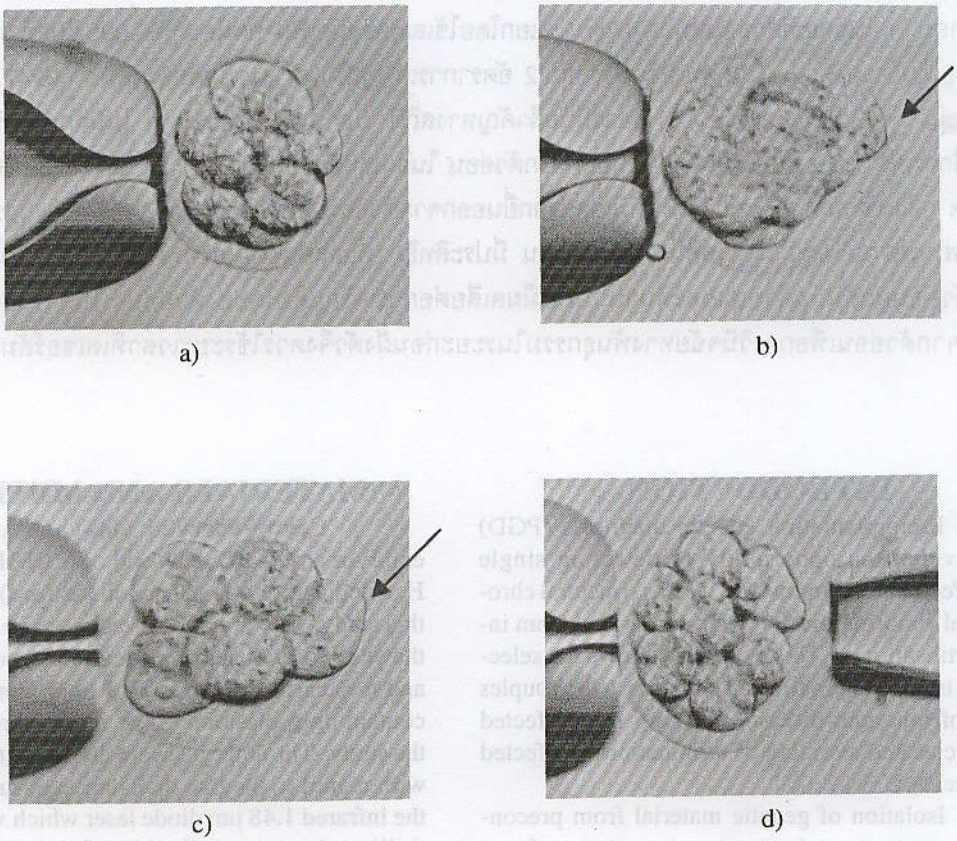

Figure 1. Laser assisted embryo biopsy
a) The embryo was immobilised by a holding pipette
b) An opening in the zona pellucida was created by the laser beam (arrow)
c) The drilled embryo with an opening in the zona pellucida (arrow)
d) A blastomere was removed by a biopsy pipette.

completely but not to harm the nearby blastomeres with the laser. The diameter of the opening was approximately two-thirds of the diameter of the blastomere to be removed. A biopsy pipette was inserted through the opening and usually one blastomere was aspirated. The biopsied embryo was transferred to the culture. After the biopsy, the embryos of both groups were subsequently evaluated for their growth on day 4 and 5 of embryonic development, using the criteria shown in Table 1.

The embryonic development on day 4 and 5, the blastocyst formation rate, the grade of the blastocysts and the hatching rate were evaluated and statistically analysed, using the epi-info programme (version 6.0). Statistical significance in this study was defined as P value ≤ 0.05.

RESULTS

Sixty-nine thawed cleavage stage mouse embryos were enrolled in the study. Twenty-two (31.9%) of which were included in group A. The number of embryos in group B was 47 (68.1%). Embryonic development on day 4 and 5, blastocyst formation rate, grade of blastocysts, and hatching rate of both groups are shown in table 2. The differences in embryonic development on day 4 and 5 between groups A and B were statistically signifi-

cant. The blastocyst formation rate of group B (46/47, 97.8%) was significantly higher than that of group A (12/22, 54.5%). No blastocyst in group A became a grade 1 blastocyst. In contrast, 35 of 46 (76.0%) blastocysts in group B were categorised as grade 1. The hatching rate of group B was 84.7%, whereas there was no hatching blastocyst in group A.

DISCUSSION

Several wavelengths of laser have been studied for their effectiveness and safety.[13,14] A laser beam that emits ultraviolet radiation and the 308 nm XeCl-excimer laser were found to cause mutagenic effects.[14,15] The erbium-yttrium aluminium garnet (Er-YAG) laser that produces infrared radiation has been thought to be safe, but this laser has to be delivered

Table 1. Criteria used for grading the embryos on day 4 and 5 of embryonic development.[11,12]

Categories	Descriptions
Degenerative	Embryos with degenerative blastomeres
Arrested	Embryos with an unchanged number of blastomeres
Morulae	Embryos in which the number of blastomeres increased until became morular shaped
Cavitating morulae	Morulae containing one or more cavities, which were initially small and eccentrically placed, and then gradually expanded to occupy most of the volume of the embryo. The cavities were possibly lined with trophectoderm or inner cell mass (ICM).
Blastocysts	Morulae developed into blastocysts, where the blastocoelic cavity was largely lined by a single layer of trophectoderm and locally by the ICM. Grade 1 : typical development, characterised by early cavitation resulting in the formation of an eccentric and then expanded cavity lined by a distinct inner cell mass and trophectoderm Grade 2 : delay in the appearance of morphological differentiation of the two-cell types of the blastocyst which usually resulted in the typical blastocyst stage being reached a day or two after initial cavitaion Grade 3 : blastocysts that on the day of formation had several degenerative foci in the ICM and the cavity collapsed within 24 hours without expanding significantly, or blastocysts that had a vacuolated appearance initially and then showed extensive degenerative foci on reaching the blastocyst stage

to the target in contact mode.[16,17] The infrared 1.48 μm wavelength laser has been reported as an ideal laser being effective, safe and not requiring contact mode.[8,10] Several studies of this laser have been performed to demonstrate its performance on both gametes and embryos of the mouse and the human.[7,9,18,19] However, more research regarding its safety and effectiveness is required prior to accepting this laser for clinical use in embryo biopsy for PGD.

In group A, the lower power level of the laser resulted in an increased pulse length of laser exposure. The length of exposure time for drilling the zona pellucida in group A was 600 ms per shot. The results of this study showed that this laser system adversely affected the developmental potential of the embryos (Table 2). All parameters which were growth development at day 4 and 5, blastocyst formation rate, grade of the blastocysts and hatching rate, in group A were adversely affected by the laser system. Compared with group B, there was a significant difference in the embryonic development between group A and B. The blastocyst formation rate, the percentage of grade 1 blastocysts obtained and the hatching rate of group B obviously increased, which was related to the shorter pulse length used in group B. The drilling mechanism of the laser system is the result of a thermal effect produced at the point of aim due to the absorption of the laser energy by the glycoprotein in the zona pellucida.[9] Although the power output used in both groups studied was similar (with different pulse length), the amount of energy reaching the specimens in groups A and B was different, 0.2 and 39 mJ, respectively. An increase in and retention of heat might elevate temperatures at localised point and then produce some damage.[20] The findings of this study clearly demonstrated the effect of a pulse length of the laser system, and confirmed the findings of Neev et al. that a lower power with a longer pulse length might cause more adverse effects than a higher power with a shorter pulse length.[15]

Table 2. Embryonic development on day 4 and 5, blastocyst formation rate, grade of the blastocysts, and hatching rate of the embryos between groups A and B.

Variables	Group A	Group B	P value
No. of embryos	22	47	-
Day 4 of embryonic development			<0.05
Arrested	16	-	
Morulae	3	31	
Cavitating morulae	3	15	
Blastocysts	-	1	
Day 5 of embryonic development			<0.05
Degenerative	6	-	
Arrested	3	-	
Morulae	1	-	
Cavitating morulae	-	1	
Blastocysts	12	46	
Blastocyst formation rate (%)	12/22 (54.5)	46/47 (97.8)	<0.05
Grade 1 Blastocysts (%)	0/12 (0.0)	35/46 (76.0)	<0.05
Hatching rate (%)	0/12 (0.0)	39/46 (84.7)	<0.05

In conclusion, a laser-assisted biopsy may be effective and safe in embryo biopsy for PGD. However, it is necessary to consider the level of laser power and the duration of exposure used in the biopsy procedure. A longer exposure period can adversely affect the developmental potential of the cleavage stage embryos. A higher power level with a shorter exposure period is suggested. Since laser assisted biopsy technique has been recently introduced, more information, therefore, is required to ensure that the new technique is effective and safe enough for using in in-vitro fertilization and PGD.

REFERENCES

1. Lissens W, Sermon K, Staessen C, Assche EV, Janssenswillen C, Joris H, et al. Review: preimplantation diagnosis of inherited disease. J Inherit Metab Dis 1996; **19:** 709-723.
2. Handyside AH, Delhanty JDA. Preimplantation genetic diagnosis; strategies and surprises. Trends Genet 1997; **13:** 270-275.
3. Delhanty JDA. Preimplantation Diagnosis: Basic science and clinical practice. Cong Anom 1998; **38:** 361-366.
4. Penketh R, McLaren A. Prospects for prenatal diagnosis during preimplantation human development. Bailliere's Clin Obstet Gynaecol 1987; **1:** 747-764.
5. ESHRE PGD Consortium Steering Committee. ESHRE Preimplantation Genetic Diagnosis (PGD) Consortium: preliminary assessment of data from January 1997 to September 1998. Hum Reprod 1999; **14:** 3138-3148.
6. 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:** 139-148.
7. Antinori S, Versaci C, Fuhrberg P, Panci C, Caffa B, Gholami GH. Seventeen live births after the use of an erbium-yttrium aluminium garnet laser in the treatment of male factor infertility. Hum Reprod 1994; **9:** 1891-1896.
8. Germond M, Nocera D, Senn A, Rink K, Delacretaz G, Fakan S. Microdissection of mouse and human zona pellucida using a 1.48 μm diode laser beam: effeciency and safety of the procedure. Fertil Steril 1995; **64:** 604-611.
9. Rink K, Delacretaz G, Salathe RP, Senn A, Nocera D, Germond M, et al. Non-contact microdrilling of mouse zona pellucida with an objective delivered 1.48 μm diode laser. Lasers Surg Med 1996; **18:** 52-62.
10. Germond M, Nocera D, Senn A, Rink K, Delacretaz G, Pedrazzini T, et al. Improved fertilization and implantation rates after non-touch zona pellucida microdrilling of mouse oocytes with a 1.48 μm diode laser beam. Hum Reprod 1996; **11:** 1043-1048.
11. Dokras A, Sargent IL, Ross C, Gardner RL, Barlow DH. The human blastocyst: morphology and human chorionic gonadotrophin secretion in vitro. Hum Reprod 1991; **6:** 1143-1151.
12. Dokras A, Sargent IL, Barlow DH. Human blastocyst grading: an indicator of developmental potential. Hum Reprod 1993; **8:** 2119-2127.
13. Rasmussen RE, Hammer-Wilson M, Berns MW. Mutation and sister chromatid exchange induction in Chinese hamster ovary (CHO) cells by pulsed excimer laser radiation at 193 nm and 308 nm and continuous UV radiation at 254 nm. Photochem Photobiol 1989; **49:** 413-418.
14. Tadir Y, Wright WH, Vafa O, Liaw LH, Asch R, Berns MW. Micromanipulation of gametes using laser microbeams. Hum Reprod 1991; **6:** 1011-1016.
15. Neev J, Gonzalez A, Licciardi F, Alikani M, Yona T, Berns M, et al. Opening of the mouse zona pellucida by laser without a micromanipulator. Hum Reprod 1993; **8:** 939-944.
16. Feichtinger W, Strohmer H, Radner KM. Erbium YAG laser for micromanipulation of oocytes and spermatozoa. Lancet 1992; **340:** 115-116.
17. Feichtinger W, Strohmer H, Fuhrberg P, Radivojevic K, Antinori S, Pepe G, et al. Photoablation of oocyte zona pellucida by erbium-YAG laser for in-vitro fertilisation in severe male infertility. Lancet 1992; **339:** 811.
18. Veiga A, Sandalinas M, Benkhalifa M, Boada M, Carrera M, Santalo J, et al. Laser blastocyst biopsy for preimplantation diagnosis in the human. Zygote 1997; **5:** 351-354.
19. Montag M, van der Van K, Delacretaz G, Rink K, van der Ven H. Laser-assisted microdissection of the zona pellucida facilitates polar body biopsy. Fertil Steril 1998; **69:** 539-542.
20. Boulnois JL. Photophysical processes in recent medical laser development: a review. Laser Med Sci 1986; **1:** 47-66.