Effect of Different Exposure Periods to The Infrared 1.48 μm Diode Laser on the Inner Cell Mass and Trophectoderm of Blastocysts

Phakphum Phophong, M.D., M.Sc.*
Darapa Manoch, M.D.*
Roungsin Choavaratana, M.D.*

Abstract : **This study was carried out to determine the effect of the duration of exposure to an infrared 1.48 μm diode laser, on the number of cells in the inner cell mass and the trophectoderm of blastocysts following laser-assisted embryo biopsy. A total of 102 mouse embryos were used in the study. The embryos were randomly divided into three groups; group A (n = 22), group B (n = 47) and group C (n = 33). The embryos in group A were biopsied using the laser with an exposure of 600 ms, whereas those in group B were biopsied using the same laser with an exposure of 5 ms. The embryos in group C were incubated in culture without any procedures, as a control group. The blastocyst formation rates of group B (46/47, 97.8%) and group C (33/33, 100%) were significantly higher than that of group A (12/22, 54.5%). The numbers of cells in the inner cell mass, trophectoderm and the total number of cells in the blastocysts in group A (16.1 ± 5.1, 35.5 ± 10.9, 51.6 ± 12.9) were similar to those in group B (14.0 ± 5.6, 36.0 ± 12.7, 50.0 ± 18.3). The numbers of cells in the inner cell mass, trophectoderm and the total number of cells in the blastocysts in group C (19.1 ± 6.5, 45.8 ± 14.0, 65.0 ± 18.7) were significantly higher than those of the study groups. In conclusion, the longer duration of exposure to the infrared 1.48 μm diode laser might adversely affect blastocyst formation. However, it might not affect the quality of the blastocysts with regard to the numbers of cells in the inner cell mass and the trophectoderm.**

เรื่องย่อ : ผลกระทบของความแตกต่างของระยะเวลาที่ตัวอ่อนถูกสัมผัสด้วยรังสีเลเซอร์ชนิดอินฟราเรดขนาดความยาวคลื่น 1.48 ไมครอนที่มีต่อจำนวนเซลล์ของอินเนอร์เซลล์แมสและโทรเฟคโตเดิร์มของบลาสโตซิสต์

ภาคภูมิ โพธิ์พงษ์ พ.บ., M.Sc.*, ดาราภา มาโนช พ.บ.*, เรืองศิลป์ เชาวรัตน์ พ.บ.*
ภาควิชาสูติศาสตร์-นรีเวชวิทยา, คณะแพทยศาสตร์ศิริราชพยาบาล, มหาวิทยาลัยมหิดล, กรุงเทพมหานคร 10700.

สารศิริราช 2545; 54: 698-703.

**Department of Obstetrics and Gynaecology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand.*

ได้ทำการศึกษาในตัวอ่อนหนูจำนวน 102 ตัว เพื่อทราบถึงผลกระทบของความยาวของช่วงเวลาที่ถูกสัมผัสด้วยรังสีเลเซอร์ชนิดอินฟราเรดขนาดความยาวคลื่น 1.48 ไมครอน ที่มีต่อจำนวนเซลล์ของอินเนอร์เซลล์แมสและโทรเฟคโตเดิร์มของบลาสโตซิสต์ที่ได้ภายหลังการแยกเซลล์บลาสโตเมียร์จากตัวอ่อนเพื่อการวินิจฉัยทางพันธุกรรมในระยะก่อนฝังตัว. ตัวอ่อนหนูจำนวน 22 ตัวถูกแยกเซลล์บลาสโตเมียร์ออกโดยใช้เลเซอร์ชนิดอินฟราเรดขนาดความยาวคลื่น 1.48 ไมครอนสัมผัสนาน 600 มิลลิวินาที (กลุ่ม เอ), ตัวอ่อนหนูจำนวน 47 ตัวถูกแยกโดยใช้เลเซอร์ชนิดเดียวกันแต่สัมผัสนาน 5 มิลลิวินาที (กลุ่ม บี), และตัวอ่อนหนูจำนวน 33 ตัวเป็นกลุ่มควบคุม (กลุ่ม ซี). พบว่าอัตราการเจริญเป็นบลาสโตซิสต์ของกลุ่ม บี (46/47, ร้อยละ 97.8) และกลุ่ม ซี (33/33, ร้อยละ 100) สูงกว่ากลุ่ม เอ (12/22, ร้อยละ 54.5) อย่างมีนัยสำคัญทางสถิติ. บลาสโตเมียร์ทั้งสามกลุ่มถูกย้อมด้วยวิธีดิฟเฟอเรนเชียลลาเบลลิงเพื่อตรวจนับจำนวนของเซลล์ของอินเนอร์เซลล์แมสและโทรเฟคโตเดิร์ม. จำนวนเซลล์ของอินเนอร์เซลล์แมส โทรเฟคโตเดิร์มและเซลล์ทั้งหมดของบลาสโตซิสต์ในกลุ่ม เอ (16.1 ± 5.1, 35.5 ± 10.9, 51.6 ± 12.9) และกลุ่ม บี (14.0 ± 5.6, 36.0 ± 12.7, 50.0 ± 18.3) ไม่แตกต่างอย่างมีนัยสำคัญทางสถิติ. จำนวนเซลล์ของอินเนอร์เซลล์แมส โทรเฟคโตเดิร์มและเซลล์ทั้งหมดของบลาสโตซิสต์ในกลุ่ม ซี (19.1 ± 6.5, 45.8 ± 14.0, 65.0 ± 18.7) สูงกว่าทั้งในกลุ่ม เอ และกลุ่ม บี อย่างมีนัยสำคัญทางสถิติ ($p < 0.05$). สรุปได้ว่า ระยะเวลาที่ตัวอ่อนถูกสัมผัสด้วยเลเซอร์ที่นานเกินไปทำให้อัตราการเจริญเป็นบลาสโตซิสต์ลดลง. ระยะเวลาที่ตัวอ่อนถูกสัมผัสด้วยเลเซอร์อาจไม่มีผลกระทบต่อจำนวนเซลล์ของอินเนอร์เซลล์แมสและโทรเฟคโตเดิร์มของบลาสโตซิสต์ที่ได้ภายหลังการแยกเซลล์บลาสโตเมียร์จากตัวอ่อนเพื่อการวินิจฉัยทางพันธุกรรมในระยะก่อนฝังตัว.

INTRODUCTION

Isolation of genetic material from preconception oocytes or embryos at various stages of preimplantation development is necessary for preimplantation genetic diagnosis (PGD). Cleavage stage embryo biopsy has been a conventional technique for most centers performing PGD.[1] Acid Tyrode's solution has been used to drill a hole in the zona pellucida of embryos, prior to removing one or two blastomeres for further genetic diagnosis. However, acid Tyrode's solution can adversely affect embryonic development as a result of its toxicity and cytoplasmic acidification leading to cytoplasmic degeneration and reduced viability.[2-4] The non-contact infrared 1.48 µm diode laser has finally been introduced and is claimed to be efficacious and safe both in vitro and in vivo.[5-7] This laser has been recently introduced for drilling the zona pellucida in PGD.[8,9] However, to date, the laser-assisted biopsy technique has not been used in most centers performing PGD.[1] More studies have to be performed to ensure its clinical efficacy and safety before it can be universally applied.

Our previous study has shown the effect of different exposure periods to the infrared 1.48 µm diode laser on preimplantation development of the embryo following cleavage stage embryo biopsy. A long duration of exposure to the laser can adversely affect the developmental potential of the biopsied embryos.[10] In this study, we further determined the effect of different exposure periods to the infrared 1.48 µm diode laser by comparing the quality of the blastocysts after the two different exposure periods. Trophectoderm (TE) and inner cell mass (ICM), which are the two main cell types of blastocyst, were evaluated and compared using a differential labelling technique.[11,12]

MATERIALS AND METHODS

Cryopreserved cleavage stage mouse embryos strain 3HI, a hybrid of C3H/HCH and 101/H, were used in this study. Freezing and thawing techniques have been described in our previous study.[10] The thawed embryos and all blastomeres were evaluated and counted. Only embryos containing more

than or equal to 6 blastomeres were used in the study. The embryos were divided into three groups group A, B and C. The embryos in group A and B were biopsied, using a laser assisted micromanipulator (Research Instruments Limited, UK) with two different periods of exposure as described previously.[10] The embryos in group A were drilled with an exposure of 600 ms, whereas the exposure period to the laser in group B was 5 ms. The embryos in group C were incubated in appropriate culture conditions for blastocyst formation, as a control group. Blastocyst formation of all groups was evaluated on day 3 after the procedure. All blastocysts derived from the three groups were sequentially processed by a differential labelling technique in order to identify and count the cell numbers of the inner cell mass (ICM) and the trophectoderm (TE).

Differential labelling technique[11,12]

The ICM and TE of the blastocysts from the three groups were differentially labelled with the polynucleotide-specific fluorochromes, propidium iodide (PI, Sigma, UK) and bisbenzimide (Hoechst No. 33258 Trihydrochloride, Sigma, UK). The zona pellucida of the blastocyst was dissolved using acid Tyrode's solution under microscopic examination. The zona pellucida-free blastocysts were rinsed in medium M2 containing 4 mg/ml bovine serum albumin. The blastocysts were incubated in rabbit anti-mouse spleen antibody (Sigma, UK), diluted 1:5 in medium M2 containing 4 mg/ml BSA for 10-15 minutes at 37°C. The blastocysts were rinsed in medium M2 containing 4 mg/ml BSA and incubated in a 1:10 dilution of guinea-pig complement serum (Sigma, UK) in medium M2 containing 4 mg/ml and 0.01 mg/ml PI at 37°C for 15-20 minutes. The blastocysts were fixed in absolute alcohol containing 0.01 mg/ml bisbenzimide for at least 1.5 hours at 4°C. The blastocysts were finally rinsed for 1 hour in absolute alcohol and mounted on microscope slides in glycerol underneath a coverslip. The blastocysts were examined using a fluorescent microscope (Nikon UFX-II, Japan). Using ultraviolet, the PI labelled-TE nuclei appeared pink and the bisbenzimide labelled-ICM nuclei appeared blue. All ICM and TE nuclei were carefully counted by drawing the outlines of the nuclei at different planes of focus through the blastocyst.

Statistical analysis

The cell numbers of the ICM and TE and the total cell numbers of the blastocysts in the three groups were recorded and statistically evaluated. Statistical analysis was performed by using epi-info programme (version 6.0). Statistical significance was defined as a p value ≤ 0.05.

RESULTS

A total of 102 cryopreserved-thawed mouse embryos were used in the study. The blastocyst formation rates of group A, B and C were 12/22 (54.5%), 46/47 (97.8%) and 33/33 (100%). The blastocyst formation rates of group B and C were significantly higher than that of group A (Table 1). Sixty-eight blastocysts from the three groups were successfully differentially labeled. The inner cell mass (ICM) and trophectoderm (TE) nuclei of the blastocysts were differentially labelled with propidium iodide and bisbenzimide. Using a fluorescence microscope, the PI labelled-TE nuclei appeared pink and the bisbenzimide labelled-ICM nuclei appeared blue, as shown in Figure 1. The numbers of cells in the ICM and the TE of the blastocysts of the three groups are presented in Table 1. The numbers of cells in the inner cell mass, trophectoderm and the total number of cells in the blastocysts in group A (16.1 ± 5.1, 35.5 ± 10.9, 51.6 ± 12.9) were similar to those in group B (14.0 ± 5.6, 36.0 ± 12.7, 50.0 ± 18.3). The numbers of cells in the inner cell mass, trophectoderm and the total number of cells in the blastocysts in group C (19.1 ± 6.5, 45.8 ± 14.0, 65.0 ± 18.7) were significantly higher than those in the groups which were treated with the laser.

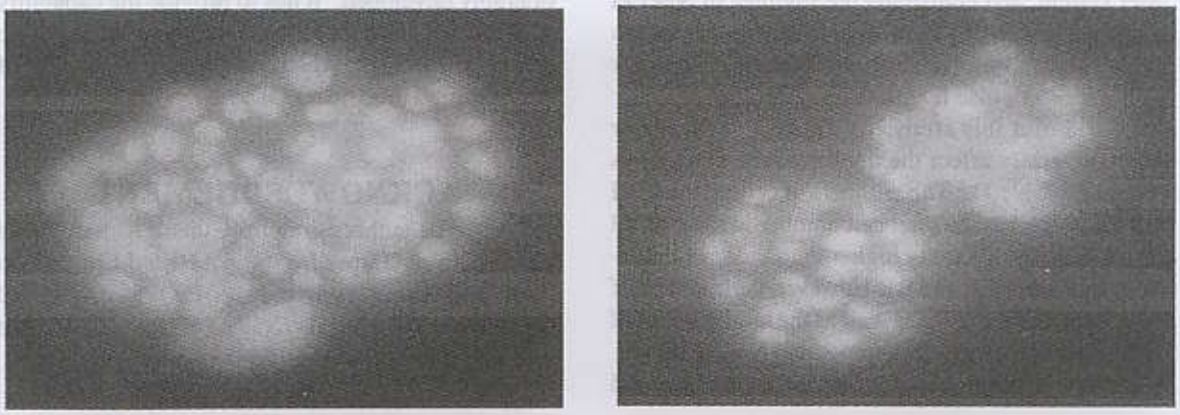
DISCUSSION

Several wavelengths of laser have been proved to be effective and safe.[13,14] Some of them, such as ultraviolet radiation and the 308 nm XeCl-excimer laser, were found to be mutagenic.[13,15] The erbium-yttrium aluminium garnet (Er-YAG) laser that produces infrared radiation has been claimed to be safe, but this laser has to be delivered to the target in contact mode.[16,17] The infrared 1.48 μm wavelength laser has been reported as an ideal laser being effec-

Table 1. Numbers of inner cell mass, trophectoderm and total cell number of the blastocysts of the three groups.

Variables	Exposure periods		Control
	600 ms (group A)	5 ms (group B)	(group C)
No. of embryos	22	47	33
Blastocyst formation (%)	12/22 (54.5)[a, b]	46/47 (97.8)[a]	33/33 (100)[b]
No. of ICM*	16.1 ± 5.1[c]	14.0 ± 5.6[d]	19.1 ± 6.5[c, d]
No. of TE*	35.5 ± 10.9[e]	36.0 ± 12.7[f]	45.8 ± 14.0[e, f]
No. of total cells*	51.6 ± 12.9[g]	50.0 ± 18.3[h]	65.0 ± 18.7[g, h]

* Mean ± SD, [a, b, c, d, e, f, g, h] p value < 0.05



a) Non-hatching blastocyst b) Hatching blastocyst

Figure 1. Differential labelling of trophectoderm (TE) and inner cell mass (ICM) nuclei of the blastocyst, using a fluorescent microscope. The propidium iodide-labelled TE nuclei appear pink, whereas the bisbenzimide-labelled ICM nuclei appear blue

tive, safe and not requiring contact mode.[17,18] Several studies of this laser have demonstrated its performance on both mouse and human gametes and embryos.[5-7] However, more research regarding its safety and effectiveness is required prior to accepting this laser for clinical use in embryo biopsy for PGD.

From our previous study, embryonic development potential was significantly decreased by a longer duration of exposure to the infrared 1.48 µm diode laser.[10] We have further studied the effect of different exposure periods to the infrared 1.48 µm diode laser by comparing the quality of the blastocysts after the two different exposure periods. The numbers of cells in the inner cell mass (ICM), trophectoderm (TE) and the total number of cells in the blastocysts following laser-assisted biopsy for two different exposure periods (group A and B) were evaluated and compared with the control group (group C). In group A, the lower power level of the laser resulted in an increased pulse length of laser exposure. The exposure time for drilling the zona pellucida in group A was 600 ms per shot. In group B, the higher power level of the laser decreased the

length of the exposure period for drilling to 5 ms. From the results of study, the blastocyst formation rate was adversely affected by the laser system (Table 1). The blastocyst formation rates of group B and C were significantly higher than that of group A. The higher blastocyst formation rate of group B was related to the shorter exposure period used. The drilling mechanism of the laser 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.[6] An increase in and retention of heat might elevate temperatures at a localised point and then produce damage to the embryos.[18] The findings demonstrated the effect of the 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.[19]

From this study, we found that the laser could adversely affect the quality of the blastocysts obtained (Table 1). The numbers of cells in the ICM and TE as well as the total cell number in the control group were significantly higher than those of the laser groups (group A and B). However, the numbers of cells in the ICM and TE as well as the total number of cells in the blastocysts of group A and B were similar. Therefore, a longer exposure period to the laser did not significantly affect the quality of the blastocysts obtained regarding the numbers of cells in the ICM and the TE. The developmental patterns of embryos, at the blastocyst formation stage, might be more sensitive to an adverse effect than the number of cells in the blastocysts.

In conclusion, laser-assisted biopsy might produce an adverse affect on the embryos. Blastocyst formation has been shown to be more sensitive to this effect. It is necessary to consider the level of laser power and the duration of exposure used in the biopsy procedure. A higher power level with a shorter exposure period has been suggested for laser-assisted biopsy. The laser system used in PGD has to be considerably developed. It has to provide the capability of dedicated control of the power level and exposure period to the laser, which can ensure the safety of laser-assisted biopsy in PGD.

ACKNOWLEDGEMENT

The authors gratefully thank Alpesh Doshi and Dr. Joyce Harper from the Assisted Conception Unit and the Department of Obstetrics and Gynaecology, University College of London, for their help and allowing this to be published.

REFERENCES

1. ESHRE PGD Consortium Committee. ESHRE Preimplantation Genetic Diagnosis (PGD) Consortium: data collection III. Hum Reprod 2002; **17:** 233-46.
2. Depypere HT, Laybaert L. Intracellular pH changes during zona drilling. Fertil Steril 1994; **61:** 319-23.
3. Garrisi GJ, Sapira V, Talansky BE, Navot D, Grunfeld L, Gordon JW. Clinical evaluation of three approaches to micromanipulation-assisted fertilization. Fertil Steril 1990; **54:** 671-77.
4. Tarin JJ, Conaghan J, Winston RML, Handyside AH. Human embryo biopsy on the 2nd day after insemination for preimplantation diagnosis: removal of a quarter of embryo retards cleavage. Fertil Steril 1992; **58:** 970-76.
5. Antinori S, Versaci C, Fuhrberg P, Panci C, Caffa B, Gholami GH. Seventeen live births after the use of an erbium-ytrium aluminium garnet laser in the treatment of male factor infertility. Hum Reprod 1994; **9:** 1891-96.
6. 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: efficiency and safety of the procedure. Fertil Steril 1995; **64:** 604-11.
7. Rink K, Delacretaz G, Salathe RP, 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.
8. Veiga A, Sandalinas M, Benkhalifa M, et al. Laser blastocyst biopsy for preimplantation diagnosis in the human. Zygote 1997; **5:** 351-54.
9. 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:** 539-42.
10. Phophong P, Doshi A, Harper JC. Preimplantation development of the cleavage stage embryo after laser assisted embryo biopsy. Siriraj Hosp Gaz 2001; **53:** 200-5.

11. Handyside AH, Hunter S. A rapid procedure for visualising the inner cell mass and trophectoderm nuclei of mouse blastocysts in situ using polynucleotide-specific fluorochromes. J Exp Zool 1984; **231:** 429-34.
12. Handyside AH, Hunter S. Cell division and death in the mouse blastocyst before implantation. Roux's Arch Dev Biol 1986; **195:** 519-26.
13. Tadir Y, Wright WH, Vafa O, Liaw LH, Asch R, Berns MW. Micromanipulation of gametes using laser microbeams. Hum Reprod 1991; **6:** 1011-16.
14. Strohmer H, Feichtinger W. Successful clinical application of laser for micromanipulation in an in vitro fertilization program. Fertil Steril 1992; **58:** 212-4.
15. 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:** 413-18.
16. Feichtinger W, Strohmer H, Radner KM. Erbium YAG laser for micromanipulation of oocytes and spermatozoa. Lancet 1992; **340:** 115-16.
17. Feichtinger W, Strohmer H, Fuhrberg P, et al. Photoablation of oocyte zona pellucida by erbium-YAG laser for in-vitro fertilisation in severe male infertility. Lancet 1992; **339:** 811.
18. Boulnois JL. Photophysical processes in recent medical laser development: a review. Laser Med Sci 1986; **1:** 47-66.
19. Neev J, Gonzalez A, Licciardi F, et al. Opening of the mouse zona pellucida by laser without a micromanipulator. Hum Reprod 1993; **8:** 939-44.