韓國家畜繁殖學會誌 22(3) : 287~292(1998)
Korean J. Animal Reprod.

Normal Pregnancy of Mouse Embryos Transferred after Assisted Hatching by a 1.48μm Diode Laser

Kim, E. Y., B. K. Yi, H. K. Nam, K. S. Lee, S. H. Yoon*, S. P. Park, K. S. Chung and J. H. Lim***

Maria Infertility Medical Institute, Seoul

1.48μm Diode Laser로 보조 부화처리 후 이식된 생쥐배의 정상임신에 관한 연구

김은영 · 이봉경 · 남화경 · 이금실 · 윤산현* · 박세필 · 정길생 · 임진호***

마리아 기초의학연구소

요 약

본 실험은 1.48μm diode laser의 인간배에 대한 적용 가능성 여부를 조사하기 위한 예비실험으로, 체외생산된 생쥐배에 1.48μm diode laser를 이용한 zona pellucida (ZP) drilling 처리가 배의 부화와 체내발달에 효과적인지를 조사하고자 실시하였다. 그 결과는 다음과 같다. 발달단계가 상이한(4-세포기배, 배반포기배) 생쥐배에 laser ZP drilling 처리한 후 72시간 (배반포기배) 또는 120시간 (4-세포기배) 동안 배양하였던 바, laser ZP drilling 처리를 받은 배반포기배의 부화율(81.8%)이 대조군 (54.2%) 이나 laser ZP drilling 처리를 받은 4-세포기배 (45.5%) 보다 유의하게 높게 나타났다 ($p<0.05$). 또한, laser ZP drilling된 배반포기배를 가임신이 유도된 대리모에 이식하였던 바, 처리군 (48.7%)의 착상율이 대조군 (43.6%)보다 약간 높게 나타났다. 한편, 임신된 대리모 일부는 분만을 유도하였던 바, 태어난 모든 새끼는 처리군에 관계없이 정상적인 염색체수 (n=40), 정상적인 성장과 생식기능을 나타내었다. 이러한 결과는 1.48μm diode laser를 이용한 ZP drilling이 생쥐배의 부화를 증진시키고 정상적인 임신을 유도할 수 있어 인간배에 대한 적용 가능성을 시사한다고 하겠다.

(Key words : Mouse IVF embryos, ZP drilling, 1.48μm diode laser, Hatching, Implantation)

I. INTRODUCTION

Artificial opening of the zona pellucida (ZP) has been proposed as a means to promote subsequent hatching of *in vitro* produced embryos (Cohen, 1991; Obruca et al., 1994). Two methods of ZP manipulation are already in use to enhance the ability of embryos to hatch: mechanical partial ZP dissection (Cohen, 1990) and chemical ZP drilling by acidic Tyrode's solution (Liu et al., 1993). Lasers are especially appreciated for their precise and atraumatic mode of action. Recently, an infrared 1.48μm diode laser beam has been shown to allow rapid, easy, non-touched microdrilling and a high degree of accuracy in the mouse or human ZP under conventional culture conditions without impairment

* 마리아 산부인과 (Maria Obs /Gyn, Seoul)
** 건국대학교 축산대학 (College of Animal Husbandry, Kon-Kuk University)

of subsequent embryo development (Germond et al., 1995; Antinori et al., 1994). In previous bovine study (Kim et al., 1998), we confirmed that embryo hatching in laser drilled group was significantly higher than that in contol group. Based on these data, this study was performed to test whether ZP drilling using a 1.48μm diode laser beam on mouse *in vitro* fertilization (IVF) embryos becomes effective the hatching and normal *in vivo* development, as a preliminary test for obtaining the additional proof that the 1.48μm diode laser could be used safely for human applications.

Ⅱ. MATERIALS AND METHODS

1. Production of mouse IVF embryos

Mouse IVF embryos were prepared as outlined by Kim et al. (1997). Oocytes were recovered from four to six weeks old F_1 female mice (C57BL/6 × CBA) which had been superovulated by intraperitoneal (i.p.) injection with 7.5IU PMSG (Sigma) and followed by i.p. injection of 7.5IU of hCG (Sigma) 50 hr later. At 13.5 hr post hCG injection, cumulus-oocyte complexes were transferred into 50μl drop of M16 medium supplemented with 0.4% BSA until insemination using epididymal sperm obtained from adult F_1 male mice (C57BL/6 × CBA). At 14 hr post hCG injection, insemination was carried out with final concentration of 1×10^6sperm/ml. Cleaved 2-cell embryos were transferred into fresh M16 medium and were further cultured for 1 day (in case of 4-cell) or 3 days (in case of blastocyst) before the treatment.

2. Experimental group

This study was carried out to examine the effect of laser ZP drilling to the hatching and implantation of *in vitro* produced mouse embryos. Firstly, to determine whether the effect of laser ZP drilling is different according to the embryonic stage, the development morphology after treatment of the laser drilled 4-cell (day 2 after IVF) and laser drilled blastocysts (day 4 after IVF) was comapared to that of non-treated hatching blastocyst (control, day 4 after IVF). Secondly, the control and laser drilled blastocysts were transferred into day 3 pseudopregnant recipients and were examined their implantation rates. Lastly, several pregnant mice of two groups were allowed to proceed to term and natural delivery and the pups were analysed chromosomal normality and tested their reproductive ability.

3. Laser ZP drilling

The set-up used for ZP microdrilling has been described in our previous report (Kim et al., 1998). Briefly, a 670nm diode laser aiming beam and the collimated 1.48μm 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 (×45) within the microscope field in a spot 8μm in diameter. The power routinely available at the image plane of the objective was 47mW corresponding to a maximal power density of 94kW/cm^2.

The early 4-cell and blastocysts to be laser drilled were suspended in groups of 5~10 in a 50μl drop of culture dish containing HEPES-buffered M2 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 2~4 milli second (ms) laser light. After treatment, the embryos were further cultured for 72 hr (in case of blastocyst) or 120 hr (in case of 4-cell) in M16 medium and observed their development morphology to hatching and hatched blastocyst.

4. Embryo transfer

Blastocysts recovered from control and laser drilling were transferred surgically on uterine horns (6~8 embryos/horn) of ICR recipient female mice on day 3 of pseudopregnancy. The day on which a copulation plug was found was designed day 1 of pseudopregnancy. Implantation rates were scored with the total number of fetuses including the resorption sites at day 15 of gestation and live birth.

5. F_2 generation follow up

Several pregnant mice of two groups (control and laser drilled blastocysts) were allowed to proceed to term and natural delivery. Development of the offspring (n= 20; control×11, laser drilled group×9) was followed over several months and their reproductive ability was established through various cross-matings between siblings.

6. Chromosome preparation and analysis

Four-week-old mice produced by embryo transfer were used in chromosome analysis. After colcemid (0.2 ml) injects i.p. for 1.5~2 hr, isolated femurs from mouse plushed two times with phosphate buffer saline. Bone marrow cells were placed in a hypotonic solution of potassium chloride (0.075M) for 25 min. and then fixed methanol : acetic acid = 3 : 1. After the slides were warmed in slide warmer for at least 24 hr, the slides were stained with 4% Giemsa at pH 6.8 and normality was scored with microscope.

7. Statistical analysis

Difference in the developmental rates between control and treatment group was compared using the Chi-square test ($p<0.05$).

Ⅲ. RESULTS

When the hatched rates of mouse embryos by laser ZP drilling were examined until 72 hr (in case of blastocyst: day 4 after IVF) or 120 hr (in case of 4-cell: day 2 after IVF) after treatment, as shown in Table 1, the data of laser drilled blastocysts (81.8%) was significantly higher than those of control (hatching blastocyst: day 4 after IVF) (54.2%) and laser drilled 4-cell embryos (45.5%) ($p<0.05$). Also, when the effect of laser drilling on implantation rates following embryo transfer in day 3 synchronized pseudopregnant recipients was examined, as indicated

Table 1. *In vitro* development of laser drilled mouse 4-cell and blastocyst stage

Treatment	Day 2 (4-cell)	Day 4 (Blstocyst)	No. (%) of development		
			24 hr later (day 5)	48 hr later (day 6)	72 hr later (day 7)
Control (HgB)		HgB*× 24	HdB × 7(29.2)	HdB ×11(45.8)	HdB × 13(54.2)[a] HgB × 11
4-cell laser drilled	35	HgB × 22	HgB × 22	HdB × 5(22.7)	HdB × 10(45.5)[a] HgB × 12
Blastocyst laser drilled		Bla. × 22	HgB × 22	HdB × 12(54.5)	HdB × 18(81.8)[b] HgB × 4

* Bla; Blastocyst, HgB; Hatching blastocyst, HdB; Hatched blastocyst.

** Means in the column without common superscripts are significantly different.

[a-b] ($P<0.05$).

Table 2. *In vivo* development of laser drilled mouse blastocysts

Repli.	Control		Laser drilled		Remark
	No. of transferred	No. of implanted	No. of transferred	No. of implanted	
1	8	3	8	5	birth
2	8	3	8	5 (+2)*	
3	12	8	11	4	birth
4	11	2 (+1)*	12	3	
Total	39	17 (43.6)	39	19 (48.7)	Control (11) Laser drilled (9)

* Parenthesis is the number of resorption sites

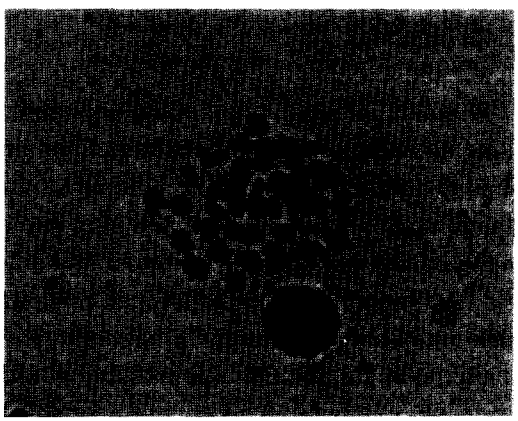

Fig. 1. The mouse chromosome of male live-born young after laser drilling. Metaphase spread showing normal chromosome complement of 40 (×800).

in Table 2, the implantation rate of laser drilled group (48.7%) was slightly higher than that of control group (43.6%). In addition, among the F_2 mice (n=20), when the several mice (control = ♂ × 4, laser drilled group = ♂ × 3) were analysed chromosomal normality and remainders were tested reproductive ability (control = ♂ × 3 and ♀ × 4, laser drilled group = ♂ × 2 and ♀ × 4) between siblings, all pups were presented normal chromosomal number (n=40, Fig. 1) and showed normal growth and reproductive ability (F_3 ; control = 22, laser drilled group = 25).

Ⅳ. DISCUSSION

In this study, we tested whether ZP drilling using a 1.48μm diode laser beam on mouse embryos becomes effective the hatching and normal *in vivo* development, as a preliminary test for obtaining the additional proof that the 1.48μm diode laser could be used safely for human applications.

Failure of ZP rupture and subsequent incomplete hatching are thought to cause implantation failure (Antinori et al., 1996). Also, prolonged exposure of human oocytes and embryos to artificial conditions appears to impair their ability to implant. Cohen (1991) demonstrated that approximately one quarter of all embryos have the ability to implant in human IVF system, that substantial number of IVF embryos are unable to breach the zona at the time of hatching, and that many can be rescued by artificial opening of those zonae. To facilitate the embryo hatching, chemical (Liu et al., 1993), mechanical (Cohen, 1990) or laser methods (Feichtinger et al., 1992) was already carried out. Especially, 1.48μm laser assisted hatching procedure presents several advantages over mechnical or chemical methods in terms of embryo survival, easiness of use and reproducibility

(Rink et al., 1994).

For use of lasers to be beneficial in embryo manipulation, the following specifications need to be met: (i) minimal thermal effect, (ii) prevention of genetic damage by using a wavelength sufficiently distant from the maximum absorption of DNA, (iii) a low ablation threshold, (iv) easy handling using existing micromanipulation. In addition, high absorption of the laser wavelength by the target is a necessary prerequisite for efficient laser ablation. To meet all these criteria, we used 1.48μm diode laser and irradiation conditions were chosen such that the holes could be drilled in the shortest time with a potentially minimal thermal effect (pulse length 2~4 ms). In the results, as shown in Table 1, the hatching arrest of *in vitro* produced blastocysts seems to be rescued by artificial opening although one shot laser drilling was not effective in early 4-cell stage. Hatched rates in laser drilled blastocysts were significantly higher than those of other two group ($p<0.05$). Those differences may be originated from the ZP thickness and the ZP elasticity between 4-cell and blastocyst at treatment. In addition, we observed that most of drilled 4-cell stage was remained in state of eight-shaped intermediate in hatching stage, as shown in other study (Rink et al., 1994; Germond et al., 1995). Also, these results indicated that hatching enhancement observed *in vitro* of laser drilled blastocysts had no an effect on the number of mice born per embryos transferred, which (48.4%) was similar to that of control (43.6%). However, there were not abnormal pattern in chromosome number, growth and reproductive ability F_2 generation in laser drilled group. In addition, we confirmed normal growth of F_3 mice in laser drilled group.

Consequently, these results demonstrated that ZP drilling using a 1.48μm diode laser can increase the embryo hatching and give rise to normal, fertile offspring.

V. SUMMARY

The objective of this study was to test whether ZP drilling using a 1.48μm diode laser beam on mouse IVF embryos becomes effective the hatching and normal *in vivo* development, as a preliminary test for obtaining the additional proof that the 1.48μm diode laser could be used safely for human applications. The results obtained in this experiment were as follows: when the hatched rates of mouse embryos by laser ZP drilling according to the embryonic stage were examined until 72 hr (in case of blastocyst: day 4 after IVF) or 120 hr (in case of 4-cell: day 2 after IVF) after treatment, the data of laser drilled blastocysts (81.8%) was significantly higher than those of control (hatching blastocyst: day 4 after IVF) (54.2%) and laser drilled 4-cell embryos (45.5%) ($p<0.05$). When the effect of laser drilling on implantation rates following embryo transfer in day 3 synchronized pseudopregnant recipients was examined, the laser drilled group (48.7%) was slightly higher than that of control group (43.6%). In addition, when the several pregnant mice delivered in two groups were analysed their chromosomal normality and tested reproductive ability, all pups were presented normal chromosomal number ($n=40$) and showed normal growth and reproductive ability. Therefore, these results demonstrated that ZP drilling using a 1.48μm diode laser can increase the embryo hatching and induce the normal pregnancy of mouse embryos.

VI. REFERENCES

1. Antinori, S., C. Versaci, P. Fuhrberg, C. Panci, B. Caffa and G. H. Gholami. 1994. Seventeen live births after the use of an

Er : Yag laser in treatment of male factor infertility. Hum. Reprod., 9:1891-1896.
2. Antinori, S., C. Panci, H. A. Selman, B. Caffa, G. Dani and C. Versaci. 1996. Zona thinning with the use of laser: a new approach to assisted hatching in humans. Hum. Reprod., 11:590-594.
3. Cohen, J., G. Wright, H. Malter, C. Elsner, H. Kort, J. Massey, M. P. Mayer and K. E. Wiemer. 1990. Impairment of the hatching process following *in vitro* fertilization in the human and improvement of implantation by assisting hatching using micromanipulation. Hum. Reprod., 5:7-13.
4. Cohen, J. 1991. Assisted hatching of human embryos. J. *In Vitro* Fertil. and Embryo transfer. 8:179-190.
5. Feichtinger, W., H. Strohmer, P. Fuhrberg, K. Radivojevic, S. Antinori, G. Pepe and C. Versaci. 1992. Photoablation of oocyte zona pellucida by erbium-yag laser for *in-vitro* fertilisation in severe male infertility. (Letter) Lancet, 339:811.
6. Germond, M., D. Nocera, A. Senn, K. Rink, G. Delacretaz and S. Faken. 1995. Microdissection of mouse and human zona pellucida using a 1.48μm diode laser beam: efficacy and safety of the procedure. Fertil. Steril., 25:604-611.
7. Kim, E. Y., S. J. Uhm, M. K. Kim, S. H. Yoon, S. P. Park, K. S. Chung and J. H. Lim. 1997. Study on epidermal growth factor (EGF) and expression of EGF-receptor (EGF-R) in mouse IVF /IVC embryo I. Additive effect of EGF and expression of EGF-R on mouse IVF embryo development. Korean J. Fertil. and Steril., 24:13-20.
8. Kim, E. Y., B. K. Yi, H. K. Nam, K. S. Lee, S. H. Yoon, S. P. Park, K. S. Chung and J. H. Lim. 1998. Enhanced rate of bovine IVM /IVF /IVC blastocyst by laser drilling. Korean J. Animal Reprod., 22:163-170.
9. Liu, H. C., N. Noyes, J. Cohen, Z. Rosenwaks and M. Alikani. 1993. Assisted hatching facilitates earlier implantation. Fertil. Steril., 60:871-875.
10. Obruca, A., H. Strohmer, D. Sakkas, Y. Menezo, A. Kogosowski, Y. Barak and W. Feichtinger. 1994. Use of lasers in assisted fertilization and hatching. Hum. Reprod., 9:1723-1726.
11. Rink, K., C. Delacretaz, R. P. Salathe, A. Senn, D. Norcera and M. Germond. 1994. 1. 48μm diode laser microdissection of the zona pellucida of mouse zygotes. Society of Photo-optical Instrumentation Engineers Proceedings Series., 2134A:412-422.

(접수일자 : 1998. 7. 31. / 채택일자 : 1998. 9. 5.)