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# Enhanced Hatching Rate of Bovine IVM/IVF/IVC Blastocyst by Laser Drilling

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# Laser Drilling 처리를 받은 체외생산된 소 배반포기배의 부화율 제고

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## 요 약

본 실험은 체외 생산된 소 배반포기배에 대한 laser drilling 처리가 배의 부화율에 미치는 영향을 조사하고자 실시하였다. 그 결과는 다음과 같다. 소 수정란의 체외 발달율을 조사하였던 바, 82.3%의 난할율 (≥2-세포기)과 체외수정 후 배양 7일째에 32.6%의 배반포 발달율을 나타내었다. 이렇게 생산된 배반포기배에 laser drilling 효과를 조사하였던 바, 처리 후 24시간째의 부화진행율(90.0%)은 대조군(44.4%)보다 유의하게 높게 나타났다($p<0.0001$). 또한, 처리 후 48시간째의 부화율(68.0%)도 대조군(33.3%)보다 유의하게 높게 나타났다. 이러한 결과는 laser drilling이 체외 생산된 소 배반포기배의 부화진행율과 부화율을 유의하게 증가시킬 수 있다는 것을 알 수 있었다($p<0.001$).

(Key words : Bovine IVM /IVF /IVC blastocyst, Laser drilling, Hatching /Hatched)

## I. INTRODUCTION

In order to improve fertilization, facilitate blastocyst hatching or perform blastomere biopsy, chemical (Tucker et al., 1993), mechanical (Malter and Cohen, 1989) or laser methods (Feichtinger et al., 1992) 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 $\mu$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 (Rink et al., 1994). Also, the drilling process in the infrared region did not affect embryo survival in mice (Germond et al., 1995) or in humans (Antinori et al., 1994). Based on these data, this study was performed to examine whether laser zona drilling on bovine IVM /IVF /IVC blastocysts becomes effective for embryo hatching.

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## Ⅱ. MATERIALS AND METHODS

### 1. Production of bovine IVM/IVF/IVC blastocyst

The culture procedures employed in the production of preimplantation embryos from bovine follicular oocytes were as outlined by Park et al. (1995). Briefly, cumulus oocyte complexes (COCs) were collected from visible follicles (2~6 mm) of ovaries, washed with TALP-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 $\mu$g/ml), estradiol-17$\beta$ (1 $\mu$g/ml), and gentamycin (25 $\mu$g/ml) at 39℃, 5% $CO_2$ incubator. After incubation for 22~24 h in IVM medium, oocytes were inseminated using highly motile sperm recovered from frozen-thawed semen separated on a discontinuous percoll column. From day 2 after IVF, cleaved embryos were cultured in m-CR1 medium. For the study, blastocysts produced *in vitro* at day 7 after IVF classified with developmental stage (Kim et al., 1996) and divided into control and laser drilled group, respectively.

### 2. ZP drilling

The set-up used for ZP microdrilling has been described in detail by Montag et al., (1998). Briefly, a 670 nm diode laser aiming beam and the collimated 1.48 $\mu$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 (×45) within the microscope field in a spot 8 $\mu$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/cm$^2$.

The classified blastocysts were suspended in groups of 5~10 in a 50 $\mu$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 milli second (ms) laser light. After treatment (Fig. 1), the embryos were fur-

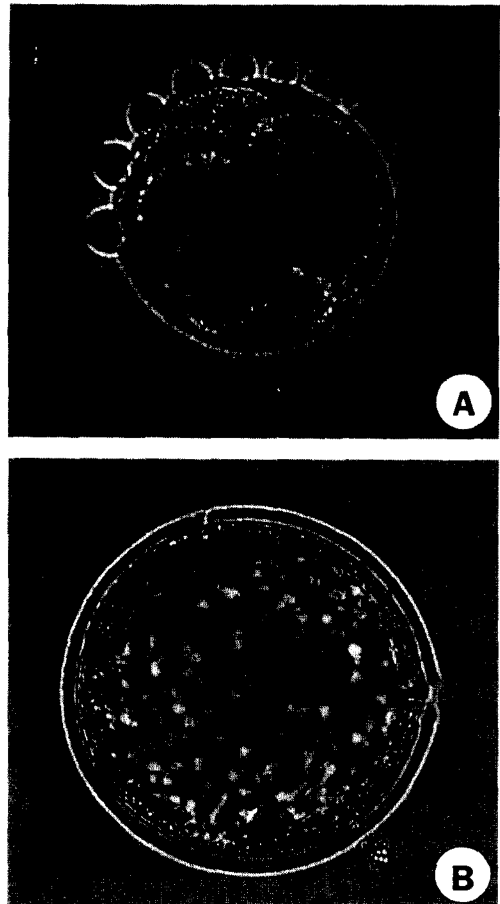


**Fig. 1. Laser drilled bovine embryos with 1.48 $\mu$m diode laser. A. Holes of different sizes are drilled by increasing the irradiation time from 3 ms to 10 ms. In this study, 3~5 ms was applied onto the classified blatocysts. B. Drilled middle blastocyst at day 7 after IVF(×300)**

ther cultured for 2 days in cumulus cell monolayered drop containing m-CR1 medium supplemented with FBS and daily observed their development morphology to hatching and hatched blastocyst. In control group also, embryo culture was same condition as treated embryo cultured.

### 3. Statistical analysis

Difference in the developmental rates between treatment group was compared using the Chi-square test.

## Ⅲ. RESULTS

The rate of *in vitro* development of bovine embryos cultured in m-CR1 medium after *in vitro* fertilization was as follows; as shown in Table 1, the average cleavage rate (≥2-cell) was 82.3% and blastocyst rate at day 7 after IVF was 32.5%. Also, development rates of classified blastocysts to early, middle, expanded and hatching stage were 41.1, 18.2, 21.0 and 9.7%, respectively, when the results were counted from total blastocyst. The results of hatching rates of bovine IVM/IVF/IVC blastocyst by laser drilling were presented in Table 2. When the hatching rates at 24 h after treatment were examined, the result of laser drilled group (90.0%) was significantly higher than that of control group (44.4%) ($p<0.0001$). Also, when the results at 48 h after treatment were investigated, the development capacity of laser drilled group (68.0%) from hatching to hatched blastocyst was significantly enhanced compared with that of control group (33.3%) ($p<0.001$).

**Table 1. Fertilization and development rates of bovine IVM/IVF/IVC embryos (r=2)**

| No. of oocytes | No. of 2-cell | Developed (%) to blastocysts at day 7 | | | | |
|---|---|---|---|---|---|---|
| | | ≥Bla.* | ErB | MB | EdB | HgB |
| 463 | 381 (82.3) | 124 (32.5) | 51 (41.1) | 35 (28.2) | 26 (21.0) | 12 (9.7) |

* Bla. ; Blastocyst, ErB; Early blastocyst, MB; Middle blastocyst, EdB; Expanded blastocyst, HgB; Hatching blastocyst.

**Table 2. Effect of laser drilling to the hatching of bovine IVM/IVF/IVC blastocyst (r=2)**

| Treatment | Day 7 blastocyst | | Developed (%) to | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 24h later | | | 48h later | | |
| | Embryo stage | Total | ≥HgB** | HgB | HedB | ≥HgB | HgB | HedB |
| Control | EdBx8 MBx14 Erx23 | 45 | 20[a] (44.4) | 14 (31.1) | 6 (13.3) | 30 (66.7) | 15 (33.3) | 15[c] (33.3) |
| Laser* drilling | EdBx9 MBx16 Erx25 | 50 | 45[b] (90.0) | 35 (70.0) | 10 (25.0) | 49 (98.0) | 15 (30.0) | 34[d] (68.0) |

* Irradiation time; 3.0~5.0 milli second

** HgB; Hatching blastocyst, HedB; Hatched blastocyst

Means in the column without common superscripts are significantly different [a-b]($P<0.0001$), [c-d]($P<0.001$).

## Ⅳ. 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 in laser system used for the gamete and embryo micromanipulation such as: 193 nm ArF-excimer laser (Palanker et al., 1991), 308 nm XeCl- excimer laser (el Danasouri et al., 1993), 2.9 μm Er:YAG laser (Stromer and Feichtinger, 1992) and so on. More recently, efficient and well controlled laser drilling of the ZP using 1.48 μm diode laser was introduced (Rink et al., 1994). 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 UV region (Neev et al., 1992). 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 with thinned ZP of blastocyst (pulse length $\leq$5 ms). As shown in Table 2, there are not any degenerated embryos through the manipulation by laser diode. 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 case of artificial micromanipulation, natural expansion of the blastocysts with thinning of the ZP does not take place (Malter and Cohen, 1989). Thus, the manipulated ZP remains relatively thick (Fig. 2D). Also, an earlier escape of the blastocyst from the ZP environment (Rink et al., 1994) and an eight-shaped intermediate stage also were observed under our *in vitro* conditions (Fig. 2C), as indicated in other study (Cohen and Feldberg, 1991). However, this study presents that hatched rates of laser drilled groups at day 8 and 9 (24 h and 48 h after treatment) were 25.0% and 68.0%, while the control groups were 13.3% and 33.3%, respectively. These results denote that ZP drilling at blastocyst stage may be strong tool to hatch embryos. Also, such positive effect was already clarified by many other researchers (Cohen et al., 1992; Germond et al., 1996).

Consequently, these results suggested that ZP drilling with a 1.48 μm diode laser can be used to enhance the hatching ability of bovine IVM/IVF/IVC blastocyst.

## Ⅴ. SUMMARY

The objective of this study was to test whether laser ZP drilling on bovine IVM/IVF/IVC blastocysts becomes effective for embryo hatching. The results obtained in this experiment were summarized as follows: When the rates of *in vitro* development of bovine embryos were examined, the average cleavage rate ($\geq$2-cell) was 82.3% and blastocyst rate at day 7 after IVF was 32.5%. Using these blastocyst, when the laser drilling effect was investigated at 24h after treatment, hatching rate of laser drilled group (90.0%) was significantly higher than that of control group (44.4%) ($p<0.0001$). In addition, at 48h after treatment, the development capacity of laser drilled group (68.0%) from hatching to hatched blastocyst was significantly enhanced compared with that of control group (33.3%) ($p<0.001$). These results de-

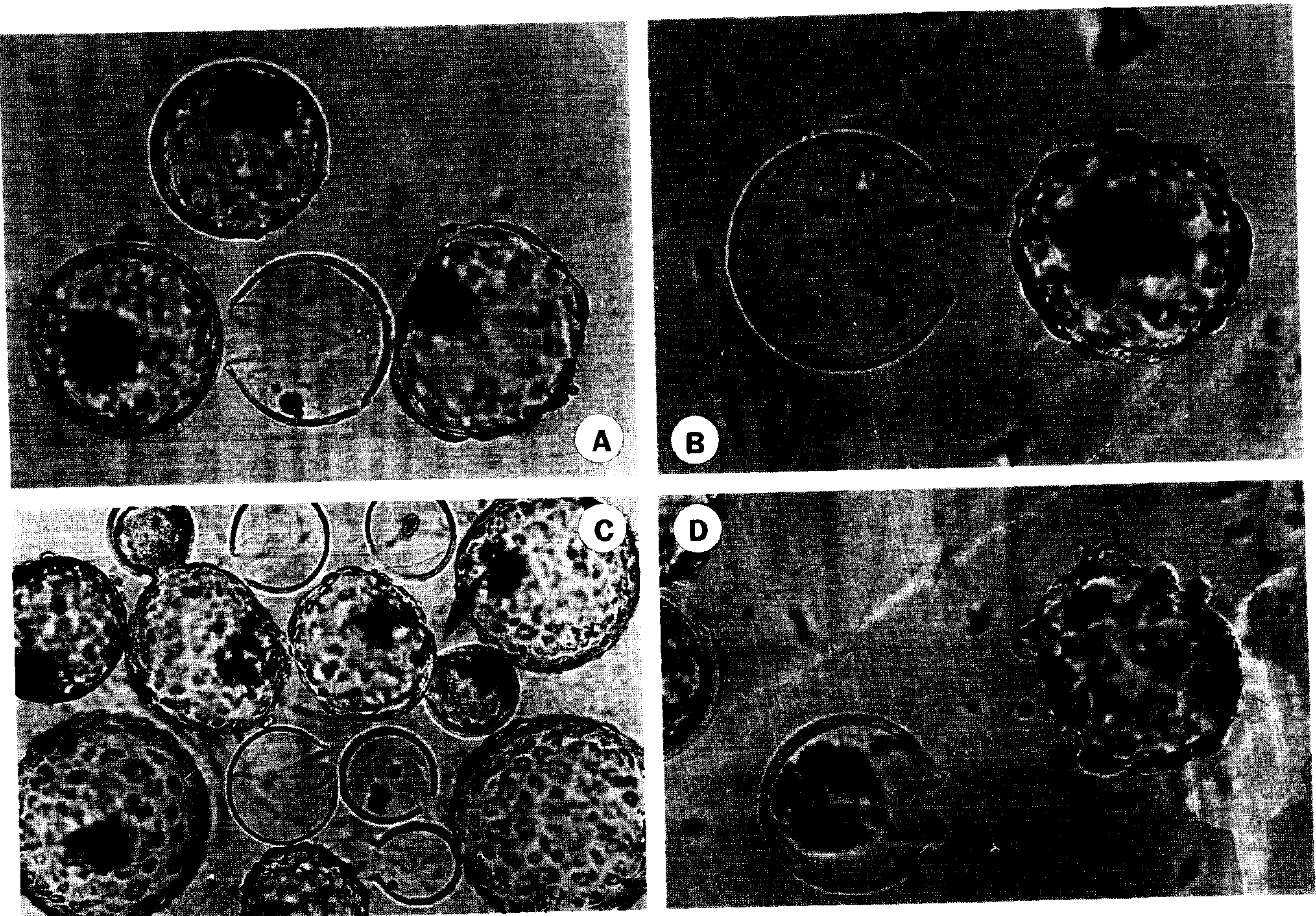


Fig. 2. *In vitro* development of drilled and non-drilled (control) bovine blastocysts. Control blastocyst development (A-B) and drilled blastocyst development (C-D). A. Control blastocysts at day 9 after IVF. (×150). B. Control hatched blastocyst. ZP has thinned. (×200). C. Drilled blastocyst 48 h after treatment. Arrow indicates characteristic eight-shaped stage during the haching process following zona drilling. (×100). D. Drilled hatched blastocyst in state of thick ZP. (×200)

monstrated that laser ZP drilling onto bovine IVM/IVF/IVC blastocysts can significantly increase the hatching and hatched rate($p<0.001$).

## 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. Cohen, J. and D. Feldberg. 1991. Effects of the size and number of zona pellucida openings on hatching and trophoblast outgrowth in the mouse embryo. Mol. Reprod. Dev., 30:70-78.
3. Cohen, J., M. Alikani, J. Trowbridge and Z. Rosenwaks. 1992. Implantation enhancement by selective assisted hatching using zona drilling of human embryos with poor prognosis. Hum. Reprod., 7:685-691.
4. el Danasouri, I., L. M. Westphal, Y. Neev, J. Gebhardt, D. Louie and M. W. Berns. 1993. Zona opening with 308 nm Xe Cl eximer laser improves fertilization by spermatozoa from long-term vasectomized mice. Hum. Reprod., 8:464-466.
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 1.48 $\mu$m diode laser beam: efficacy and safety of the procedure. Fertil. Steril., 25:604-611.
7. Germond, M., A. Senn, D. Nocera, K. Rink and G. Delacretaz. 1996. Assisted hatching of frozen-thawed embryos with a 1.48 $\mu$m diode laser enhances pregnancy outcome in patients who had several previous nidation failures. Ameri. Socie. for Reprod. Medi., Abstract: P-051.
8. Kim, E. Y., S. J. Uhm, S. E. Kim, S. H. Yoon, S. P. Park, K. S. Chung and J. H. Lim. 1996. ICM-trophectoderm cell numbers of bovine IVM/IVF/IVC blastocysts J. Animal. Reprod., 20(1):27-34.
9. Malter, H. E. and J. Cohen. 1989. Partial zona dissection of the human oocyte: a non-traumatic method using micromanipulation to assist zona pellucida penetration. Fertil. Steril., 51:139-145.
10. Montag, M., K. Van der Ven, G. Delacretaz, K. Rink and H. Van der Ven. 1998. Laser-assisted microdissection of the zona pellucida facilitates polar body biopsy. Fertil. Steril., 69(3):539-542.
11. Neeve, J., Y. Tadir, P. Ho, M. W. Berns, R. H. Asch and T. Ord. 1992. Microscope-delivered ultraviolet laser zona dissection: principles and practices. J. Assist. Reprod. Genet. 9:513-23.
12. Palanker, D., S. Ohad, A. Lewis, A. Simon, J. Shenkar S. Penchas and N. Laufer. 1991. Technique for cellular microsurgery using the 193nm eximer laser. Lasers Surg. Med., 11:580-586.
13. Park, S. P., S. E. Kim, S. J. Uhm, E. Y. Kim, T. Kim, S. H. Yoon, K. S. Chung and J. H. Lim. 1995. Effect of simple serum-free medium, CR1, on the development of IVM/IVF bovine embryos. Kor. J. Fertil. Steril., 22(2):105-108.
14. Rink, K., C. Delacretaz, R. P. Salathe, A. senn, D. Norcera, and M. Germond. 1994. 1.48 $\mu$m diode laser microdissection of the zona pellucida of mouse zygotes. Society of

Photo-optical Instrumentation Engineers Proceedings Series. 2134A:412-422.

15. Strohmer, H. and W. Feichtinger. 1992. Successful clinical application of laser for micromanipulation in an *in vitro* fertilization program. Fertil. Steril., 58:212-214.
16. Tucker, M. J., N. M. Luecke, S. R. Wiker and G. Wright. 1993. Chemical removal of the outside of the zona pellucida of day 3 human embryos has no impact on implantation rate. J. Assist. Reprod. Genet., 10: 187-191.

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