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Effects of Media Volume on Blastocyst Formation, Cell Numbers and ICM Proportion in Mouse Two-cell Embryos

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배양액 용량이 마우스 2-세포기 배의 배반포 형성, 세포수 및 내세포괴 비율에 미치는 영향

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연구 목적: 본 연구의 목적은 마우스에서 배양액의 용량이 배반포 배 형성과 세포수에 미치는 영향을 조사하기 위하여 실시하였다.

연구 재료 및 방법: 3~4주령 ICR 암마우스에게 48시간 간격으로 5 IU PMSG와 hCG 주사 후 (hCG 주사 후 수컷과 동숙) 46~50시간에 난관으로부터 총 138개의 2-세포기 배를 회수하여 2 ml (group I) 또는 50 μl (group II)의 배양액 (Dulbecco's Modified Eagle Medium + 20% human follicular fluid)에서 72시간 동안 배반포기까지 배양하였다. 배반포 배는 zona-intact (ZiB)와 zona-escape (ZeB)로 등급을 구분하고 나서, propidium iodide와 bisbenzimide를 이용한 differential staining 방법으로 염색하여 평균 세포수, 내세포괴 (ICM) 세포수, 영양배엽 (TE) 세포수, 총 세포수에 대한 ICM의 비율 (%ICM) 및 ICM:TE 비율을 조사하였다. 결과에 대한 유의성 검정은 χ^2 test와 *t*-test를 이용하였으며, $p<0.05$일 때 통계적인 차이가 있는 것으로 하였다.

결 과: Group I과 II에서, 총 배반포 (62.3±20.7% vs. 63.8±22.9%), ZiB (31.9±24.0% vs. 30.4±18.2%)와 ZeB 형성율 (30.4±20.8% vs. 33.3±22.3%)은 차이가 없었다. 87개의 배반포 배를 염색 시도하였는데, 명확하게 differential staining된 41개의 배반포 배만을 대상으로 세포수를 조사하였다. 평균 세포수 (61.6±19.5 vs. 63.7±26.8), ICM 세포수 (13.0±10.6 vs. 12.8±10.5), TE 세포수 (49.0±19.0 vs. 47.8±18.7), %ICM (21.0±12.6% vs. 21.1±13.2%) 및 ICM:TE 비율 (1:3.77±4.9 vs. 1:3.72±4.8)에서도 group I과 II에서 차이가 없었다.

결 론: 마우스에서 배 발생 능력의 척도로 쓰이는 배반포 배 형성율, 배반포 배의 등급, 세포수 및 %ICM 등이 20% 난포액을 첨가한 MEM 배양액의 용량에 따라 영향을 받지 않았다.

Key Words: Media volume, Mouse blastocyst, Mean cell number, ICM and TE cell number, ICM proportion

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Human zygotes are cultured either single or groups, sometimes in a relatively large volume (about 2 ml) of culture medium.[1~7] However several studies[8~13] have suggested that *in vitro* development of mouse embryos is enhanced by increasing the number of embryos per volume of culture medium. The cleavage rate and viability of the mammalian preimplantation embryo is greatly reduced by culture *in vitro*,[14~16] indicating the present culture systems are far from optimal. The formulations of conventional embryo culture media do not reflect either the ionic[17,18] or the metabolite[19] composition of luminal fluid within the female reproductive tract. Furthermore, the physical conditions of the oviduct and uterus are not mimicked, and so during culture any paracrine factor(s) secreted or synthesized by the female tract will be absent from the *in vitro* system. In addition, embryos are often cultured in relatively large volumes of medium compared to the sub-microliter amounts of fluid present in the lumen of the reproductive tract.[11] Therefore, any autocrine factor(s) produced by the embryo will be greatly diluted during culture *in vitro*.[8,9,20] Thus, it is conceivable that irrespective of the suitability of the culture medium, physical factors, such as media volume, may become a limiting parameter in the culture system.

The aim of this study was to investigate whether media volume in culture of 2-cell embryos had an effect on blastocyst formation, cell numbers and inner cell mass (ICM) proportion in mice.

MATERIALS AND METHODS

1. Experimental design

Two different media volumes (2 ml and 50 μl) were used for *in vitro* culture of mouse 2-cell embryos to blastocyst stage.

2. Preparation of media

1) Culture media

Two cell embryos were incubated in Dulbecco's Modified Eagle Medium (MEM, 119660-025, Gibco, USA) containing 20% human follicular fluid (hFF) until the blastocyst stage at 37℃ in a humidified atmosphere of 5% CO_2 for 72 hrs.

2) Differential staining media

Blastocysts were stained in solution 1 and 2.

(1) Solution 1

Phosphate buffered solution (PBS) with 1% Triton X-100 (T-9254, Sigma, USA) and 100 μg/ml propidium iodide (PI, P-4170, Sigma, USA).

(2) Solution 2

Fixative solution of absolute ethanol (99.9%, Duksan, Korea) with 25 μg/ml bisbenzimide (Hoechst 33258, B-2883, Sigma, USA).

3. Preparation of 2-cell embryos and *in vitro* culture

Female, 3~4 week-old, mice (ICR) were employed to induce ovulation by intraperitoneal injection of 5 IU hCG (CG-10, Sigma, USA) followed 5 IU PMSG (G-4877, Sigma, USA) injection after 48 hrs. Female mice were naturally mated with male, 10~15 week-old mice (ICR), and checked for a vaginal plug at 16~18 hrs after hCG injection. Females were sacrificed by cervical vertebrae dislocation at approximately 48 hrs treatment of hCG. Two cell embryos (Figure 1A) were flushed from the dissected oviducts of the sacrificed mice and put into experimental media (2 ml: group I; 50 μl: group II). Recovered 2-cell embryos were rinsed three times in experimental media and cultured until blastocyst stage and assessed for morphology (zona-intact blastocyst: early ~ expanded, ZiB, Figure 1B; zona-escape blastocyst: partially hatching ~ completely hatched blastocyst, ZeB, Figure 1C, D)

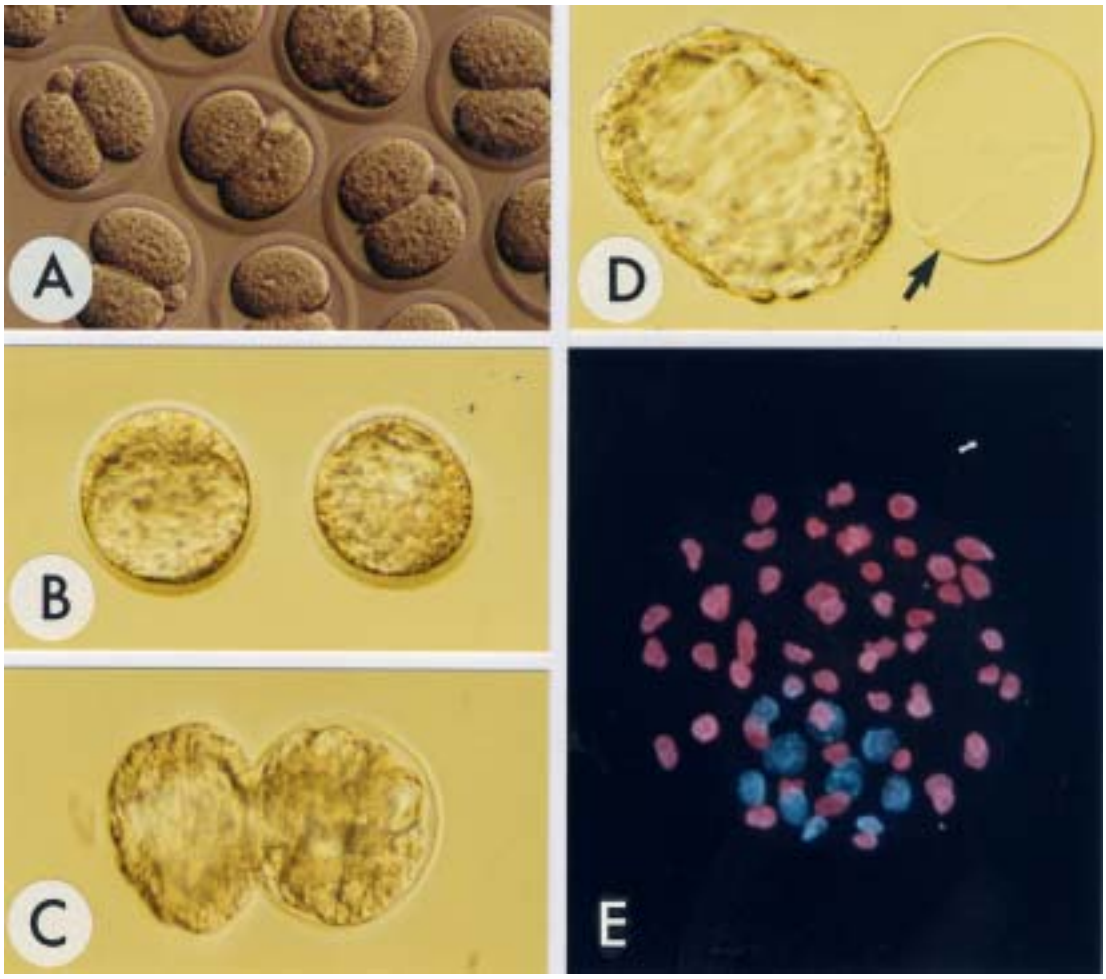

Figure 1. Specific features of the developing embryos and differential staining of blastocysts in mouse (×200). **(A)** Retrieved 2-cell embryos. **(B)** Zona-intact blastocyst (ZiB). **(C)** Zona-escape blastocysts (ZeB): hatching blastocyst from zona pellucida (ZP). **(D)** Completely hatched bla- stocyst from ZP (arrow) (ZeB). **(E)** Differentially stained blastocyst. Note that the intense pink color represents the chromatin in nuclei of lysed trophectoderm cells that had been both red (PI) and blue (bisbenzimide). Inner cell mass nuclei remain blue, because these cells were not permeabilized **(E)**.

at approximately 72 hrs after culture.

4. Differential staining of blastocyst

Blastocysts were stained differentially by the Park method.[21] Briefly, blastocysts were incubated in 2 ml of solution 1 briefly (8~15 sec), and then immediately transferred into solution 2 and stored at 4℃ (≥1.5 hrs). Fixed and stained blastocysts were transferred from solution 2 directly into glycerol without excess amount of solution 2. Blastocysts with glycerol were mounted onto a slide glass in a drop of glycerol, gently flattened with a cover slide, and visualized for cell counting. Cell counting was performed on an fluorescent microscope (BX-50, Olympus, Japan) equipped with an ultraviolet lamp and excitation filters. The intense pink color represents the chromatin in nuclei of lysed

Table 1. Effects of media volume on blastocyst formation and their cell numbers in mice

	Volume of media*	
	2 ml (Group I)	50 μl (Group II)
No. of examinations	6	6
No. of 2-cell embryos used	69	69
No. of blastocysts (%)		
Total	43 (62.3±20.7)	44 (63.8±22.9)
Zona-intact (ZiB)	22 (31.9±24.0)	21 (30.4±18.2)
Zona-escape (ZeB)	21 (30.4±20.8)	23 (33.3±22.3)
No. of blastocysts stained	41	44
Cell numbers		
Total	2,524	2,801
Mean	61.6±19.5	63.7±25.8
Range	13~125	8~162
No. of blastocysts differentially stained	23	18
Cell numbers (mean)		
Total	1,427	1,091
ICM	299 (13.0±10.6)	231 (12.8±10.5)
TE	1,128 (49.0±19.0)	860 (47.8±18.7)
% ICM of total cells	21.0±12.6	21.2±13.2
ICM:TE ratio	1:3.77±4.9	1:3.72±4.8

*No significant differences within both groups by a combination of Chi square and *t*-test.
The % of blastocyst formation, ICM and ICM:TE between groups were compared by a χ^2-test.
Differences in the cell numbers of blastocyst between groups were assesed by *t*-test.
Values are mean ± SD.

trophectoderm cells that had been both red (PI) and blue (bisbenzimide). Inner cell mass nuclei remain blue, because these cells were not permeabilized (Figure 1E).

5. Statistical analysis

Data obtained from the experiments [blastocyst formation rates, cell numbers (mean, ICM and TE), %ICM of total cells and ICM:TE ratio] were analyzed using Sigma Plot 2001 (Version 7.0) and Microsoft Excel 2002. Differences were analyzed by a combination of Chi square and *t*-test. Results were considered statistically significant when P values were less than 0.05.

6. Photographs

Photographs were taken with a inverted microscope equipped with a Hoffman contrast-modulation system (Diaphot 300, Nikon, Japan) or fluorescent microscope equipped with UV green filter system (BX 50, Olympus, Japan) and camera systems using Kodak film (MAX, ASA 400).

RESULTS

The purpose of this study was to investigate the effect of two different volume of media on blastocyst formation and their cell number in mouse.

Two different media volumes (2 ml, group I; 50 μl, group II) were used for *in vitro* culture of mouse 2 cell embryos. A total of 138 two cell embryos was used in either group I (n=69) or group II (n=69). The results of blastocyst formation and their cell numbers are summarized in Table 1.

In group I and group II, no differences were found in the number of total blastocyst formation (62.3±20.7%, 43/69 vs. 63.8±22.9%, 44/69), ZiB (31.9±24.0%, 22/69 vs. 30.4±18.2%, 21/69), ZeB (30.4±20.8%, 21/69 vs. 33.3±22.3%, 23/69).

Staining of 87 blastocysts (group I: n=43; group II: n=44) was attempted. Two blastocysts were lost during processing (in group I), and 85 blastocysts (group I: n=41; group II: n=44) were stained. In 44 blastocysts (group I: n=18; group II: n=26), careful examination in whole mount suggested that some of TE and/or ICM nuclei had not been differentially labelled. And blastocysts stained differentially were 41 (group I: n=23; group II: n=18). No differences were found in mean cell number (61.6±19.5, 2524/41 vs. 63.7±25.8, 2801/44), ICM cell number (13.0±10.6, 299/23 vs. 12.8±10.5, 231/18), TE cell number (49.0±19.0, 1128/23 vs. 47.8±18.7, 860/18), %ICM of total cells (21.0±12.6%, 299/1427 vs. 21.2±13.2%, 231/1091) and ICM:TE ratio (1:3.77±4.9, 299:1128 vs. 1:3.72±4.8, 231:860) in group I and group II.

DISCUSSION

We examined the effects of media volume of *in vitro* culture on the developmental capacity of mouse embryos. The results showed that no differences were found in the blastocyst formation tare and cell numbers in different media volume (2 ml and 50 μl) (Table 1). Lane and Gardner[11] reported that decreasing the incubation volume (from 320 to 20 μl) significantly ($p<0.01$) increased blastocyst cell number and embryos development after transfer. And increasing the number of embryos incubated per drop (from 1 to 16) significantly increased the number of 2 cell embryos reaching blastocyst stage in 5 or 320 μl in mouse.[11] Salahuddin *et al.*[12] suggested that increasing the embryo density (1, 5, 10 or 20 embryos) in 20 μl drops significantly increased the rates of embryos reached hatched blastocyst stage in *in vitro* fertilized mouse embryos. Larson and Kubisch[20] demonstrated that culture in groups increased the formation of blastocysts, the percentage of hatching blastocysts and the production of interferone-τ in *in vitro* matured, fertilized and cultured bovine oocytes. Using mouse embryos, Canseco *et al.*[10] also reported that the developmental score at 120 hrs for embryo cultured in 10 μl drops was significantly higher than that for embryos cultured in 20 μl or 40 μl. Paria and Dey[9] speculated that embryos produce growth factors which could act in an autocrine fashion to stimulate development. In single embryo culture these factors would be too diluted to be effective; however, the inferior development of single cultured embryos should be overcome by addition of appropriate growth factors, such as epidermal growth factor (EGF), transforming growith factor (TGF)α and TGFβ1. In contrast, using IVF embryos, Keefer *et al.*[22] failed to observe significant improvement in blastocyst development and cell number with the addition of EGF and TGFβ1. These results indicate species differences in the types of growth factors

acting in autocrine and paracrine fashions. It is therefore important to examine the type of growth factors affecting the development of human embryos when we apply these results in humans.

Basically, hFF contains in any of molecules that exert diverse biological activities including embryonic growth factors and cytokines. Therefore, it should be emphasized that in our experiment, supplementation of 20% hFF to the media may be sifficient to compensate the lack of paracrine or autocrine interactions among cultured embryos in group I and II.

In conclusion, blastocyst formation, cell numbers and ICM proportion were not affected by the media volume in mouse 2-cell embryos. This study has implications for clinical work, where *in vitro* fertilization and subsequent embryo culture conducted in relatively large or small volumes of media, and where embryos are grown individually or groups in media.

REFERENCES

1. Park KS, Choi IK, Lee JS, Song HB. The effects of glutamine on blastulation of human embryos on vero cells in vitro. Kor J Fertil Steril 1998; 25: 65-70.
2. Park KS, Lee TH, Choi IK, Song HB, Chun SS. Comparison of blastulation and pregnancy rates of fertilized human oocytes obtained after conventional *in vitro* fertilization and intracytoplasmic sperm injection. J Mamm Ova Res 2000; 17: 51-7.
3. Park KS, Song HB, Chun SS. Late fertilization of unfertilized human oocytes *in vitro* fertilization and intracytoplasmic sperm injection cycles: conventional insemination versus ICSI. J Assist Reprod Genet 2000; 17: 419-24.
4. Park KS, Song HB, Lee TH, Chun SS. Subsequent embryo transfers (SET) on day 2 and day 5: it's safety and effectiveness. Kor J Fertil Steril 2000; 27: 165-72.
5. Chun SS, Park KS. Birth of a healthy infant after *in vitro* fertilization and embryo transfer in patient of total uterine prolapse. J Assist Reprod Genet 2001; 18: 346-8.
6. Lee HJ, Park KS, Song HB, Lee TH, Cho YL, Chun SS. Comparison of embryonic developmental capacity by different co-culture time of oocytes in IVF-ET cycles. Kor J Fertil Steril 2002; 29: 21-2.
7. Kim DW, Park KS, Song HB, Lee TH, Chun SS. The clinical outcomes after embryo transfer (ET) on day 2 and day 5 or subsequent ET on day 2-5, 2-6, 2-7, 3-5 and 4-7 in *in vitro* fertilization-ET cycles. Kor J Fertil Steril 2003; 30: 31-7.
8. Wiley LM, Yanami S, Van Muyden D. Effect of potassium concentration, type of protein supplement and embryo density on mouse preimplantation development *in vitro*. Fertil Steril 1986; 45: 111-9.
9. Paria PC, Dey SK. Preimplantation embryo development *in vitro*: cooperative intractions among embryos and role of growth factors. Proc Natl Acad Sci USA 1990; 87: 4756-60.
10. Canseco RS, Sparks AET, Pearson, RE, Gwazdauskas FC. Embryo density and medium volume effects on early murine embryo development. J. Assist Reprod Genet 1992; 9: 454-7.
11. Lane M, Gardner DK. Effect of incubation volume and embryo density on the development and viability of preimplantion mouse embryos *in vitro*. Hum Reprod 1992; 7: 558-62.
12. Salahuddin S, Ookutsu S, Goto K, Nakanishi Y, Nagata Y. Effects of embryo density and co-culture of unfertilized oocytes on embryonic development of *in-vitro* fertilized mouse embryos. Hum Reprod 1995; 10: 2382-5.
13. Park KS, Lee TH, Chun SS, Song HB. Effects of medium on blastocyst formation, cell number and percentage of ICM in mice. Kor J

Fertil Steril 2002; 29: 237-43.
14. Bowman P, MaLaren A. Cleavage rate of mouse embryos *in vivo* and *in vitro*. J Embryol Exp Morphol 1970; 24: 203-7.
15. Harlow GM, Quinn P. Development of preimplantation mouse embryos *in vitro* and *in vivo*. Aus J Biol Sci 1982; 35: 187-93.
16. Streffer C, Van Beuningen D, Molls M, Zanboglou N, Schulz S. Kinetics of cell proliferation in the preimplantation mouse embryo *in vivo* and *in vitro*. Cell Tissue Kinet 1980; 13: 135-43.
17. Borland RM, Hazra S, Biggers JD, Lechene CP. The elemental composition of the environments of the gemetes and preimplantation embryo during the initiation of pregnancy. Biol Reprod 1977; 16: 147-57.
18. Borland RM, Biggers JD, Lechene CP, Taymer ML. Elemental composition of fluid in the human fallopian tube. J Reprod Fertil 1980; 58: 479-82.
19. Gardner DK, Leese HJ. Concentrations of nutrients in mouse oviduct fluid and their effects on embryo development and metabolism *in vitro*. J Reprod Fertil 1990; 88: 361-8.
20. Larson MA, Kubisch HM. The effects of group size on development and interferon-τ secretion by *in-vitro* fertilized and cultured bovine blastocysts. Hum Reprod 1999; 14: 2075-9.
21. Park KS, Park SB, Lee TH, Chun SS, Song HB. Effects of exposure of propidium iodide and bisbenzimide on differential staining of mouse blastocysts. Kor J Fertil Steril 2002; 29: 317-22.
22. Keefer CL, Stice SL, Paprocki AM, Golueke P. *In vitro* culture of bovine IVM-IVF embryos: coorperative interaction among embryos and the role of growth factors. Theriogenology 1994; 41: 1323-31.