Comparison of Cell Numbers in Inner Cell Mass vs Trophectoderm and Establishment of ES-like Cells Derived from Day 7 to 9 IVF Bovine Blastocysts

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소 체외수정 유래 7~9일령 배반포배에서 내세포괴 대 영양배엽세포의 비율 및 배아간세포 확립율의 비교

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SUMMARY

포유류 배반포배의 epiblast는 내세포괴에 포함되어 있으며 이 epiblast cells이 배 및 태아의 생식세포와 일반 체세포로 분화된다 (Beddington, 1986; Lawson 등, 1991). 그런데 조기에 발달된 부화배반포기 배가 지연발생된 부화배반포기 배보다도 많은 epiblast cells을 가지고 있다고 한다(Talbot 등, 1995). 그래서 본 연구에서는 체외수정 유래의 배반포배의 발육속도에 따른 내세포괴 / 영양배엽세포의 비율 및 배아간세포 확립 효율을 비교하여 발달일령 간에 차이가 있는지를 규명하고자 하였다.

공시한 소의 난포란을 TCM-199에 0.5 μg / ml FSH, 5 μg / ml LH, 10% FBS, 100 units / ml penicillin, 및 100 μg / ml streptomycin을 첨가하여 39℃, 5% CO_2 조건하에서 24시간 동안 체외성숙한 후, 5 μg / ml heparin으로 수정능이 획득된 1×10^6 sperm / ml의 정자로 체외수정을 유도하였다. 체외수정후 18~20시간에 과립막세포를 vortexing으로 제거하여 얻은 모든 체외수정란을 3 mg / ml BSA, 20 μl / ml NEM amino acids, 40 μl / ml BME amino acids, 10 mM glycine 및 1 mM alanine이 함유된 CR1aa 배양액에서 BRL 단층세포와 공배양을 실시하였다. 수정후 7, 8 및 9일째 (체외수정일 : 0일)에 확장배반포기까지 발달한 수정란을 이중염색 및 배아간세포의 확립 실험에 공시하였다.

체외수정후 24시간에 분할된 총 1,145개의 수정란이 7, 8 및 9일째에 후기 배반포기까지 각각 222 (15.6%), 103 (7.2%) 및 52 (3.6%)로 발달하여 총 377개 (26.4%)가 발달하였다. 내세포괴 / 영양배엽세포의 비율은 7일 및 8일째 배반포배에서 각각 47.2±11.9 / 95.1±24.4개 (33 / 67%) 및 40.3±12.4 / 83.3±26.9개 (33 / 67%)로서 9일째 배반포배의 19.3±8.1 / 62.3±23.1개 (24 / 76%) 보다 유의적 (P<0.05)으로 높았다. ES-like cells을 확립하기 위하여 후기배반포기 배를 mouse embryonic fibroblast 단층 공배양기에 옮긴 후 5일에 내세포괴의 부착 여부를 판정하고, 10일에 배아간세포의 확립 여부를 판정하였다. 그 결과 7, 8 및 9일째의 배반포기배의 각각 47.7% (82 / 172), 30.9% (22 / 71) 및 15.6% (5 / 32)에서 배아간세포가 확립되었다.

이상의 결과에서 배반포기 까지의 발육이 빠른 수정란에서 영양배엽에 대한 내세포괴세포의 비율이 높았고 배아간세포의 확립율도 높다는 사실을 입증할 수 있었으며, 이와 같은 결과에서 체외수정란 유래 배반포배의 질을 결정할 수 있을 것으로 생각된다.

I. INTRODUCTION

A culture system has been established for in vitro production of bovine embryos using follicular oocytes collected from the isolated ovaries (Xu et al., 1987; Fukui et al., 1988; Goto et al., 1988; Moore & Bondioli, 1993). In general, it has been reported that the pregnancy rate was lower for bovine embryos derived from *in vitro* fertilization than for those recovered *in vivo*. However, the factors causing this difference have not fully been determined.

The inner cell mass of the mammalian blastocyst contains a core of cells termed the epiblast. The epiblast gives rise to the somatic and germ cells of the embryo and fetus (Beddington, 1986; Lawson et al., 1991). Two distinct cell lineages arise with the formation of the blastocyst. They are: 1) the inner cell mass (ICM) cells /pluripotent cells that will differentiate into all tissues of the developing fetus, and 2) the trophectoderm (TE) cells, epithelial-like cells responsible for fluid transport during blastocyst development and later for attachment to the uterine endometrium and formation of the fetal placenta. The distribution of cells to these cell lineages is vital for future development, and their morphology has been useful for the evaluation of embryo culture systems for several species (Papaioannou & Ebert, 1988; Hardy et al., 1989; Iwasaki et al., 1990; Erbach et al., 1994; Giles & Foote, 1995). For an undisturbed embryonic development, a sufficient cell number of inner cell mass is essential.

ES cells were totipotent, or pluripotent, and could be reintroduced into the mouse preimplantation morula or blastocyst where they subsequently contributed to somatic and germinal tissues of the developing mouse. Talbot et al. (1995) demonstrated that the early hatching blastocyst was a richer source of epiblastocy-derived cell lines than late-hatching blastocysts and that *in vivo* produced bovine blastocysts were more efficient sources of epiblasts than IVMFC derived blastocysts. Jiang et al. (1992) found that both the total cell number and ICM cell number in grade I / II blastocysts were significantly higher than in grade III or IV / V blastocysts and the late developing blastocysts were of poor quality as judged by the cell number.

The objective of this study was to determine the differences in the cell number ratio of ICM to TE and establishment of ES-like cells between the Day 7, 8 and 9 IVMFC bovine blastocysts.

II. MATERIALS AND METHODS

1. Oocyte maturation and fertilization

Bovine ovaries were obtained from a local slaughter house. Cumulus-oocyte complexes were aspirated from 2 to 8 mm diameter antral follicles with an 18 gauge hypodermic needle, selected for an envelope of compact and complete cumulus cells, and then washed three times in HEPES-buffered Tyrode's medium (Fissore et al., 1992) supplemented with 3 mg /ml bovine serum albumin (BSA), 0.2 mM pyruvate, 100 units /ml penicillin, 100 μg /ml streptomycin, then oocytes were transferred into 500 μl TCM 199 (M199: HyClone laboratories) maturation medium containing 10% FCS, 0.5 μg /ml bovine FSH, 5 μg /ml bovine LH, 100 units /ml penicillin, 100 μg /ml streptomycin in 4-well culture dishes (Nunc, Holland) and cultured at 39℃ in a humidified atmosphere of 5% CO_2 in air for 24 hours. At 24 hrs after initiation of maturation,

spermatozoa were prepared for *in vitro* fertilization according to a method described by Parrish et al. (1988). Cryopreserved semen was thawed in 37℃ water and placed in 10 ml m-PBS for washing and centrifugation, and then capacitated with 400 μl of 50 ng/ml heparin solution for 15 minutes at 39℃. The capacitated sperm were diluted with TL-FERT medium to approximately 1×10^6 sperm/ml in drops containing the oocyte.

2. *In vitro* embryo development and evaluation

Embryos were co-cultured with 50 to 60% confluent BRL cell (ATCC #1442) at 39℃ in CR1aa medium (Moore & Bondioli, 1993) containing 3 mg/ml BSA, 20 μl/ml NEM amino acids, 40 μl/ml BME amino acids, 10 mM additional glycine, and 1 mM additional alanine in 5% CO_2 in air for 24 hrs. All of the cleaved embryos were then counted, removed, and transferred to a new culture dish. Embryos were transferred onto fresh BRL monolayers with fresh medium every 3 days. All embryos that reached the expanded blastocyst stage at day 7, 8, and 9 after insemination (insemination: day 0) were recovered for differential staining or establishment of ES like-cells.

3. Differential staining for counting ICM and TE cells

The procedure of Papaioannou and Ebert (1988) or Giles and Foote (1995) for differential staining of inner cell mass and trophectoderm cells with propidium iodide (CalbioChem) and brobenzimide (Hoechst 33342; Sigma) were carried out with minor modifications. Intact embryos with a zona pellucida were treated with 0.5% (w/v) pronase (Type XXX; Sigma) in PBS plus 1% (v/v) FBS for 2 min to remove the zona pellucida. Embryos were washed and transferred to 50 μl of rabbit anti-bovine serum (antibody, Sigma B8270) diluted 1:5 with TCM-199, incubated for 40 min under 5% CO_2 in air at 39℃. They were then in PBS containing 0.5% polyvivylperolidium (PVP) to removed anti-serum and transferred to 50 μl of Guinea pig complement (Gibco) diluted 1:5 in TCM-199. After 30 min of incubation at 39℃, embryos were briefly washed in PBS to remove the serum and complement and transferred to a 2~3 μl drop of glycerol on a glass slide. They were examined as a whole mount without a coverslip and then gently squashed with a coverslip, and cell were counted. Intact or ICM cells are permeable to the Hoechst stain and nonpermeable to propidium iodide and therefore fluoresce blue. The TE or lysed cells are permeable to both stains and fluoresce pink. Whole mounts of stained embryos revealed a blue mass (ICM) surrounded by pink nuclei (TE) when illuminated by 380 nm light.

4. Establishment of ES like-cells of bovine blastocysts

The expanded blastocysts were exposed to 0.5% pronase solution supplemented with 0.5% PVP to remove the zonapellucidae. The ICMs were then seperated the TE dissection with 32 gauge needle. The isolated ICMs were cultured on EF feeder cells. Attachment of ICMs or epiblasts were identified by morphology at day 5, 10 of culture. A transformed EF cell line obtained from 14 to 16 day old mouse fetuses were used as a feeder layer. EF cells were propagated in Dulbecco's modified Eagle's medium with high glucose + L-glutamin, 10% FBS, and antibiotics as above (10% DMEM). EF cells were prepared as described by Robertson (1987) using 5 μg/ml mitomycin C (Sigma) for mitotic inactivation. Bovine blastocyst derived cell cultures were maintained in a 1:1 mixture of DMEM (low glu-

cose) and M199 containing 10% FBS, 0.1 mM Z -mercaptoethanol, NEM, L-glutamine, antibiotics, and nucleotide supplementation as described by Robertson (1987). Initial ES like-cell colonies were observed by 6 to 7 days of culture. Only cells with epithelial-like morphology, small cuboidal size, small cytoplasmic /nuclei ratio, and cytoplasmic vesicles were judged as ES like-cells.

5. Statistical analysis

All the analyses were performed using StaView v4.01 software (Abacus Concepts, berkeley, CA).

Ⅲ. RESULTS AND DISCUSSION

1. Embryo development competence and proportion of ICM vs TE cells

Embryos produced *in vitro* were co-cultured for 9 days in the presence of BRL cells. As shown in Table 1, 80.3% of IVF oocytes (1,145/ 1,426) were cleaved at 48 hrs after insemination. All the cleaved embryos were co-cultured with BRL monolayers in CR1aa medium to develop to blastocyst stage for 9 days. Among the 1,145 cleaved embryos, a total of 377 embryos (26.4 %) were developed to blastocyst and the days of blastocyst formation were varied as Day 7

Table 1. Development competence of bovine IV-MFC embryos

Stage achieved	No. of embryos (%)
Inseminated	1,426
Cleaved	1,145(80.3)
Day-7 blastocysts	222(15.6)[a]
Day-8 blastocysts	103(7.2)[b]
Day-9 blastocysts	52(3.6)[c]
Total blastocysts	377(26.4)

* Values with different superscripts were significantly different (P<0.05).

(222 embryos: 15.6%), Day 8 (103 embryos: 7. 2%), and Day 9 (52 embryos: 3.6%). About 60% of 377 blastocysts were developed to expanded blastocyst at Day 7. BRL monolayers in CR1aa medium used in this experiment were effective for embryo development *in vitro*. Reed et al. (1996) reported that BRL cells could be used for co-culture of bovine IVF embryos. Although these cells alone were less effective than combination with BOE cells, they were easier in preparation and maintenance. Compared with the co-culture system with BOE cells, BRL cell monolayers were easier in maintenace and better in culture results, if they were maintained in recommended media such as Ham's F-12 (Watanabe and Ide, 1993), Coon's modified Ham's F-12 (Coon and Weiss, 1969), or Dulbecco's modified Eagle's medium (Marquart et al., 1981).

All the embryos reached the expanded blastocyst stage at Day 7, 8 and 9 after insemination were recovered and stained for examining the ICM vs TE ratio or total cell numbers as shown in the Table 2 and Fig. 1. The analysis revealed that the ratio of ICM vs TE cell number in day 7 and 8 blastocysts {47.2 vs 95.1 (33:67%) and 40.3 vs 83.3 (33:67%)} was significantly (P<0. 05) higher rather than in day 9 blastocysts {19.2 vs 62.3 (24:76%)}. Also the ICM and TE cell number or total cell numbers in day 7 and 8 blastocysts have greater (P<0.05) numbers of cells compared to day 9 blastocysts in terms of the average number. The cell number might be a valid indicator of the viability of preimplantation embryos although morphological criteria alone were not valid indicators (Papaioannou and Ebert, 1988). Jiang et al. (1992) mentioned the existence of a variation in cell number by different grades of blastocysts derived from IVM, IVF and IVC bovine oocytes, and clearly indicated that later developing blastocysts were of poor quality as judged by the cell number. The

Table 2. Cell numbers and proportion of ICM vs TE of bovine blastocyst

Days to blastocyst formation	No. of blastocysts stained	No. (%) of nuclei*		
		ICM	TE	Total
7	50	47.2±11.9(33)[a]	95.1±24.4(67)[b]	142.3±34.4(100)[a]
8	31	40.3±12.4(33)[a]	83.3±26.9(67)[b]	123.6±38.3(100)[a]
9	19	19.3± 8.1(24)[b]	62.3±23.1(76)[a]	81.6±29.6(100)[b]

* (Mean±S.D.), Different superscripts within the same column were significantly (P<0.05) different.
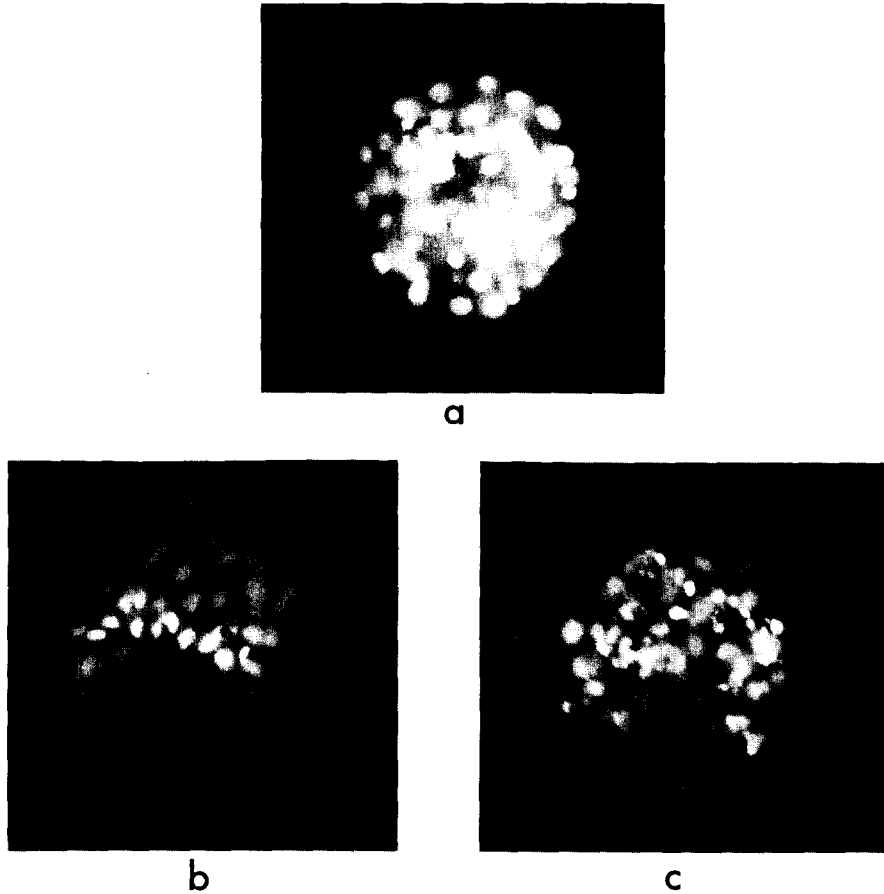

Fig. 1. Differential staining of bovine blastocyst after 7, 8 and 9 days culture *in vitro* in A, B, and C, respectively (250×).

embryo developing quickly to the blastocyst stage was of higher quality than one that developed later, although these were placed in the same catagory. It was clearly demonstrated that the reduced proportion of the ICM expressed a reduced viability (Willadsen and Polge, 1981).

2. Establishment of ES-like cells by Day 7, 8, and 9 blastocyst

To establish of ES like-cells on the EF monolayers, embryos developed to expanded blastocysts at day 7, 8, and 9 were placed on EF monolayers and checked it up to attach a ICM colony at day 5 and then keep culturing to day 10 for determining of establishment of ES like-cells. As shown Table 3 and Fig. 2, the establish-

Table 3. Establishment of ES like-cells from bovine blastocysts

Days to blastocyst formation	No. of blastocysts used	No. of ES-like cell colonies established (%)
7	172	82(47.7)[a]
8	71	22(30.9)[b]
9	32	5(15.6)[b]

* Values with different superscripts were significantly (P<0.05) difference.

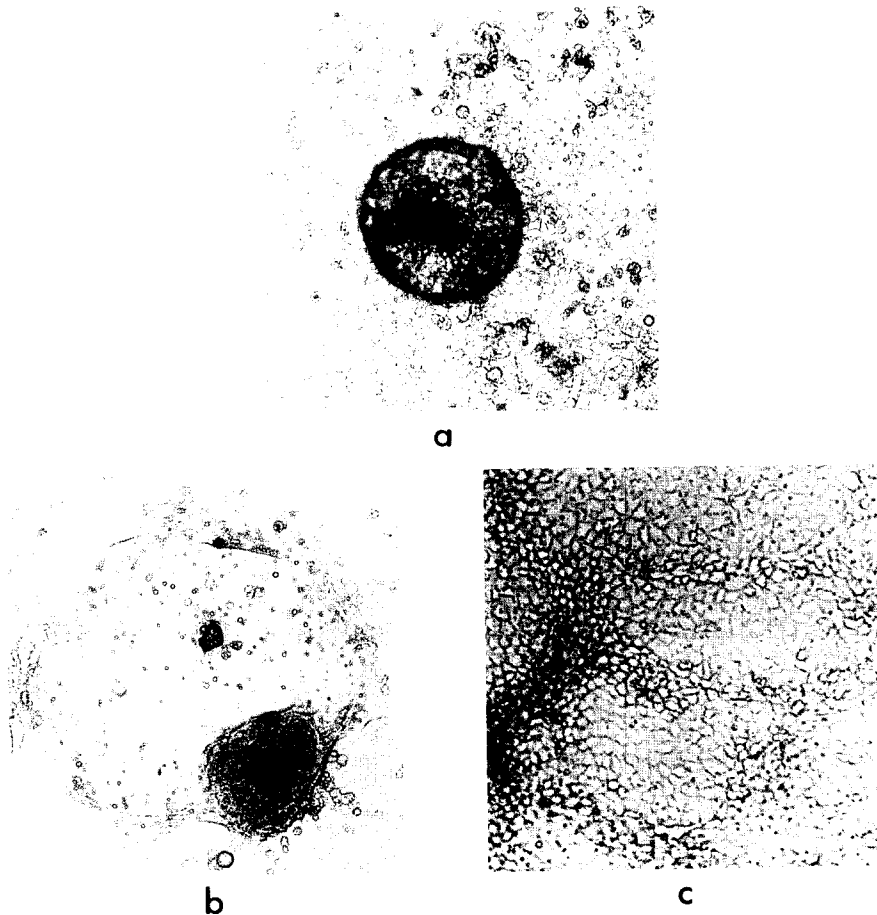Fig. 2. ES-like cell colony following 5, 10 and 15 days (a, b and c) culture of a dissected bovine blastocyst, respectively (250×).

ment rates of ES like-cells at day 7 blastocysts (47.7%) were significantly (P<0.05) higher rather than in day 8 and 9 blastocysts (30.9 and 15.6%). There is not significantly difference between day 7 ad 8 blastocysts. The early growing blastocysts are a richer sourse of epiblast-derived cell lines than late-growing blastocysts. Talbot et al. (1995) reported that ICM colony formation depended jointly on the age and the source of the blastocyst with day 8 ICMs from *in vivo* blastocysts producing colonies at nearly twice the rate (90%) observed *in vitro* blastocysts (47%). Embryos with more cells have higher quality scores and this was correlated with better development after transfer (Dorland et al., 1988). Fewer cells in IVMFC blastocysts may also explain the lower production of epiblasts and cell lines from day 9 hatching blastocysts. Jiang et al. (1992) reported that the late developing IVMFC blastocysts had fewer cells than early developing blastocysts. Consequently, the reduced cell proliferation of ICM cell vs TE cells may be the cause of the low establishment of ES like-cells of blastocysts derived from *in vitro* fertilization.

Ⅳ **SUMMARY**

This study was carried out to determine the effect of days of blastocyst development on the cell ratio of inner cell mass (ICM) to trophectoderm (TE) and possibility of establishment of ES like-cell colonies of blastocysts derived from *in vitro* matured, fertilized, and cultured (IVMFC) bovine embryos. Bovine oocytes were cultured in TCM-199 supplemented with 0.5 μg/ml FSH, 5 μg/ml LH, 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 39℃ in humudified atmosphere of 5% CO_2 for 24 hrs and then inseminated with sperm suspension of 1×10^6 sperm/ml containing 5 μg/ml heparin. At 18~

20 hrs after insemination, cumulus cells were removed by vortexing. All embryos were then cultured in CR1aa media containing 3 mg/ml BSA, 20 μl/ml NEM amino acids, 40 μl/ml BME amino acids, 10 mM glycine and 1 mM alanine over BRL monolayer cells. All the embryos that reached the expanded blastocyst stage at day 7, 8, and 9 after insemination (insemination : day 0) were either differentially stained or used to establish ES like-cells. A total of 841 ova cleaved by 48 hrs after insemination {222 (15.6%), 103 (7.2%), and 52 (3.6%)} developed to expanded blastocyst stage by day 7, 8, and 9, respectively. The ratio of ICM vs TE cell number in day 7 and 8 blastocysts 47.2±11.9 vs 95.1±24.4 (33/67%) and 40.3±12.4 vs 83.3±26.9 (33/67%) was significantly (P<0.05) higher rather than in day 9 blastocysts 19.2±8.1 vs 62.3±23.1 (24/76%). Also the ICM and TE cell number or total cell numbers in Day 7 and 8 blastocysts have greater (P<0.05) numbers of cells compared to day 9 blastocysts in terms of the average number. To induce establishment of ES like-cells, expanded blastocysts were transferred onto EF monolayer and attachment of ICMs was counted at Day 5 and establishment of ES like-cells at day 10. After 10 days in culture, 82/172 (47.7%), 22/71 (30.9%), and 5/32 (15.6%) had established ES like-cells for day 7, 8, and 9 blastocysts, respectively.

Results indicated that a higher ratio of ICM to TE cells and improved establishment of ES-like cells resulted from embryos that developed more quickly to blastocyst stage, and ICM to TE cell ratio and establishment of ES-like cells might affect or determine the quality of blastocysts derived from IVMFC bovine blastocysts.

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