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ICM-Trophectoderm Cell Numbers of Bovine IVM/IVF/IVC Blastocysts

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체외성숙, 수정 및 체외배양에서 생산된 소 배반포기배의 ICM과 Trophectoderm세포수에 관한 연구

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요 약

본 연구는 immunosurgery와 polynucleotide-specific 형광물질을 이용한 differential labelling기법으로 체외에서 소 수정란의 배발달을 유기하는데 효과적인 것으로 알려진 CR1배양액을 사용하여 체외생산된 소 배반포기배의 inner cell mass (ICM)와 trophectoderm (TE)의 총 세포수를 조사하고자 실시하였다. 공시 배반포기배는 체외수정 후 8일째에 얻어졌다. 체외생산된 배반포기배는 배반포강의 확대와 투명대 두께의 감소를 기준으로 초기, 중기 및 팽윤단계로 구분하였으며, 또한 같은 배발달군내의 배반포기배는 다시 두 군으로 나누어 bisbenzimide만을 처리하여 얻어진 총세포수와 immunosurgery와 two polynucleotide-specific 형광물질을 이용하여 얻어진 ICM와 TE의 총세포수를 비교하여 얻어진 결과는 다음과 같다. 1) 체외수정 후 8일째 배반포기배의 발달율은 29.3%였으며, 초기, 중기, 팽윤 및 부화단계로 구분하였을 때의 발달율은 각각 8.7, 9.9, 7.6, 3.1% 였다. 2) Bisbenzimide를 이용한 배반포기배의 총 세포수는 초기, 중기 및 팽윤단계가 각각 46.9±8.6, 66.2±12.5, 122.8±14.4를 나타냈다. 이러한 결과는 CR1이 소 수정란의 발달에 적절한 배양액임을 알 수 있었다. 3) Immunosurgery와 polynucleotide-specific 형광물질을 이용한 differential labelling기법으로 배반포기배의 ICM과 TE 세포수를 초기, 중기 및 팽윤단계로 나누어 조사한 결과, ICM 세포수는 각각 12.8±5.9, 26.3±8.4, 35.5±15.0개 이었고, TE 세포수는 각각 30.5±5.0, 41.3±8.2, 81.1±13.4개로 나타나 ICM과 TE 세포수는 초기 배반포기배에서 팽윤 배반포기배로 진행됨에 따라 두배에서 세배 정도 증가되었음을 알 수 있었다. 또한, differential labelling과 bisbenzimide기법에서 얻어진 각각의 총세포수를 비교하였을 때 총세포수는 발달의 진행 정도에 따라 증가되며 그와 동시에 동일한 군 간의 세포수도 거의 유사함을 알 수 있었다. 따라서, ICM과 TE를 differential labelling하는 기법은 수정란의 quality를 평가하는데 매우 유용한 기법으로서 착상전 embryo 발달을 연구하는데 효과적으로 이용될 수 있다는 것을 시사한다.

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Ⅰ. INTRODUCTION

In spite of the diversity of eutherian mammals, their early preimplantation development is similar. However, investigations in the mechanisms of control of early morphogenesis have been done mainly in the mouse, other mammalian species including those of economically important agricultural animals are increasingly the subject of experimental work. Papaioannou and Ebert (1988) suggested that the cell number may be a valid indicator of the viability of preimplantation embryos although morphological criteria alone are poor indicators. Iwasaki *et al.* (1990) suggested that the proportions of ICM cells in bovine blastocysts from *in vitro* fertilization decreased gradually with advance in development although a relatively large variation was found in the data within a category and also indicated that the reduced cell proliferation of ICM cells of blastocyst cultured *in vitro* after *in vitro* fertilization than that of blastocysts cultured in rabbit oviducts after *in vitro* fertilization or blastocysts cultured *in vivo* may be the cause of the low pregnancy rates of blastocysts derived from *in vitro* fertilization. Therefore, study on cell number and allocation of ICM and TE cells in later preimplantation stage can be used as an indicator of embryo quality according to culture condition and treatment variables (Iwasaki *et al.*, 1994a, b; Ray *et al.*, 1995; Conaghan *et al.*, 1993). On the other hand, the investigation of numbers of inner and outer cells in morula and blastocyst was first studied in the mouse by Tarkowski (1966) using Giemsa staining. This technique allowed the measurement of inside and total cell numbers, with average outside cell numbers obtained by subtraction. Embryo cell number has also been studied by embryo fixation and sectioning (Copp, 1978, 1979; Fleming *et al.*, 1987). Recently, Handyside and Hunter (1984) established a rapid and simple method using immunosurgery and two polynucleotide-specific fluorochromes. The ICM and TE nuclei are differentially labelled with two fluorochromes which exhibit widely different fluorescent spectra and then be counted under fluorescent light. Thus, the propidium iodide (PI) -labelled TE nuclei appeared pink or red; the bisbenzimide-labelled ICM nuclei appeared blue or unlabelled.

The objective of this study was to examine the cell number of Total, ICM and TE cells of bovine blastocysts according to development progression cultured in CR1 medium, which was reported as successfully supporting medium for preimplantaion bovine embryo development to the blastocyst stage (Rosenkrans *et al.*, 1993), by differential labelling of the nuclei with immunosurgery and polynucleotide-specific fluorochromes.

Ⅱ. MATERIALS AND METHODS

1. Bovine blastocyst production

Bovine blastocysts were produced by *in vitro* fertilization of *in vitro* matured oocytes (Park *et al.*, 1995). Briefly, follicular oocytes were collected into TL-Hepes with 0.1% bovine serum albumin (BSA; Fraction V, Sigma) from the ovaries of Holstein cows obtained from a local slaughterhouse. The cumulus oocytes complexes were cultured in TCM-199 maturation medium, which was prepared with 50μl drop, supplemented with FBS (10%, Gibco), sodium pyruvate (0.2mM), FSH (1μg/ml), estradiol-17β(1μg/ml) for 22~24hr at 39℃ in CO_2 incubator. For fertilization, matured oocytes were washed with Sp-TALP and transferred to fertilization drops (Fert-TALP). Highly motile bull spermatozoa were recovered from frozen-thawed semen

separated on a discontinuous percoll gradient, resuspended at a concentration of 2.5×10^7 cells/ml and added with 2μl (5×10^4 cells/50μl drop) into 44μl fertilization drops together with 2μl of heparin (2μg/ml) and 2μl of PHE stock. Fertilization was assessed as cleavage rate (≥ 2-cell) after 44±2h coincubation with the sperm. For *in vitro* culture, cleaved embryos were cultured in CR1 medium supplemented with fatty acid-free BSA (FAF-BSA, 3mg/ml) and then cultured in changed CR1 medium supplemented with 10% FBS every 48h. Blastocysts were obtained at day 8 after *in vitro* fertilization.

2. Blastocysts classification

Blastocysts were classified to early, middle, expanded stage according to the developmental morphology; blastocoel expansion and zona thickness (Fig. 1). Also, blastocysts in the same category were divided into two parts to check the Total cell number by using bisbenzimide only and ICM, TE and Total cell number by using immunosurgery and two polynucleotide-specific fluorochromes.

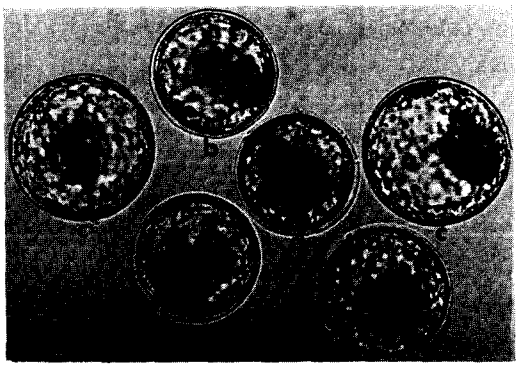

Fig. 1. Morphology of bovine blastocysts; the stage of development was determined as follows a) Early blastocysts; blastocoel is smaller than 2/3 of the whole embryonic cell, b) Middle blastocysts; blastocoel is larger than 2/3 of the whole embryonic cell, c) Expanded blastocysts; blastocoel is larger than 2/3 of the whole embryonic cell and thinning of the zona pellucida.

3. Total cell count

For the count of total blastomeres, the classified blastocysts were fixed with 2% formalin solution for 2~3min. prior to bisbenzimide (No. 33342, 2.5μg/ml, Sigma) treatment. Fixed blastocysts were washed, placed on a slide glass and covered with slip and then pressed down the edges until formation of sample for the good observation. And then an appropriate bisbenzimide solution (about 50μl) dropped beside the coverslip, filled the square under coverglass with the sol. and sealed the edges with fingernail polish. Observation was carried out under ultra violet filter incorporated fluorescent microscope on day 1 after making sample.

4. Differential labelling of ICM and TE nuclei

Differential cell counts were carried out by the method of Hardy *et al.* (1989) with some modifications. Briefly, TE nuclei are labelled first specifically with fluorochrome PI (Sigma). This fluorochrome is excluded from viable ICM cell but labelled TE cells undergoing antibody-mediated complement lysis during immunosurgery. The whole embryo is rapidly fixed and both the ICM and TE nuclei labelled with bisbenzimide. The protocol was as follows; embryo zona was removed in 0.5% pronase (Sigma) sol. and allowed to recover for 10min in TL-Hepes. Embryos were placed on ice for 15min. in 15mM trinitrobenzene sulfonic acid (TNBS; Sigma) to label cell surface proteins with covalently bound trinitrophenol (TNP) groups. This allowed the use of an antiserum against dinitrophenol (DNP) which crossreacts with TNP-labelled proteins. Embryos were then washed completely

and incubated in 0.1mg /ml anti-DNP-BSA (ICN Immunological.) in TL-Hepes for 10min at 39℃. Embryos were again washed sufficiently in TL-Hepes and then incubated in 0.01mg /ml PI and 15% (v /v) guinea pig complement (Sigma) in TL-Hepes for 20~30min. at 39℃. This step resulted in the lysis of the TE cells and the red staining of TE nuclei. Immediately after this step, embryos were placed in absolute alcohol containing the fluorochrome bisbenzimide (0.05mM, Sigma). After overnight storage at 4℃, the embryos were washed in absolute alcohol for at least 1hr, and mounted in glycerol under a coverslip on a slide glass. Labelled nuclei were observed under ultra violet excitation filter incorporated fluorescent microscope and counted TE nuclei labelled with PI and bisbenzimide appeared pink or red, ICM nuclei labelled with bisbenzimide appeared blue or unlabelled.

5. Statistical analysis

Difference in number of cells between development groups was compared using the Student's t-test.

Ⅲ. RESULTS AND DISCUSSION

1. Development of preimplantation bovine embryos

The rate of *in vitro* development of bovine embryos cultured in CR1 medium after *in vitro* fertilization was as follows; as shown in Table 1, the average cleavage rate was 82.6% and blastocyst rate at day 8 after *in vitro* fertilization was 29.3%. Also, development rates of classified blastocysts to early, middle, expanded and hatching stage were 8.7, 9.9, 7.6 and 3.1%, respectively.

Culture of bovine embryo has been difficulty imposed by the 8-cell block (Camous *et al.*, 1984). However, many researchers reported that the use of trophoblastic vesicle (Camous *et al.*, 1984), cumulus cell (Goto *et al.*, 1989) and oviductal cell (Eyestone and First, 1989) for emryo co-culture has been beneficial effect for the development of bovine embryos. Our results about the development of bovine embryos cultured in CR1 medium were similar with those from co-culture of them. At this viewpoint, CR1, chemically defined medium can be used as a basic medium in studying the role of embryotrophic factors (Park *et al.*, 1995).

2. Total cell number of bovine IVM/IVF /IVC blastocyst

The counts of total blastomere by using bisbenzimide were presented in Table 2. and Fig. 2A. In this experiment, total cell count by using

Table 1. Fertilization and development rates of bovine IVM/IVF/IVC embryos

No. of oocytes	No. of cleaved	Development rate(%) of bovine blastocysts				
		≥Blastocyst	Early	Middle	Expanded	Hatching
512	423(82.6)	124(29.3)	37(8.7)	42(9.9)	32(7.6)	13(3.1)

Table 2. Total cell number of bovine blastocysts cultured in CR1 medium by using bisbenzimide

Blastocyst stages	No.	Cell number
Early	15	46.9± 8.6(38~57)[a]
Middle	18	66.2±12.5(54~92)[b]
Expanded	15	122.8±14.4(104~154)[c]

[a,b,c] Means in the same column without common superscripts are significantly different (P<0.01)

bisbenzimide was carried out to compare with the total cell number counted from ICM and TE number by using differential labelling (Fig. 2B). As shown in Table 2, total cell numbers of classified blastocysts to early, middle and expanded were 46.9±8.6, 66.2±12.5 and 122.8±14.4, respectively. These results showed that total cell numbers were increased with development level and averaged almost three doublings between the early blastocyst and expanded blastocyst stage. Also, these results indicated that the cell number of blastocyst cultured in CR1 medium *in vitro* was very high when compared with the cell number of blastocyst produced *in vitro* obtained by Iwasaki et al (1990). Analysis of variance by Student's t-test indicated that the mean cell number of blastocysts was significantly different among the development progression ($P<0.01$).

Total cell number and mitotic index at the blastocyst stage are frequently examined as a means of comparing cleavage rate in control and treated embryos (Handyside and Hunter, 1984). Papaioannou and Ebert (1988) indicated that there was a good correlation between cell number and morphological development although cell number within each morphological category varied widely and there was considerable overlap between categories. Also, Iwasaki *et al.* (1990) demonstrated that large variation within a category may be caused by a variation in the quality of embryos and that an embryo developing quickly blastocyst stage was higher quality than one that developed later although these were placed in the same category. Cavitation and early blastocoel formation of bovine embryos on day 8 may be interpreted as delayed development. However, some blastocysts degenerated in the course of development and those poor blastocysts were not included in order to count accurately according to development advance level. Although a few blastocysts were examined, blastocyst cell number showed a trend of increase with expansion and exhibited differences in the same category with healthy level.

3. Numbers of ICM and TE cell of bovine IVM/IVF/IVC blastocyst

The count of ICM and TE cell number by us-

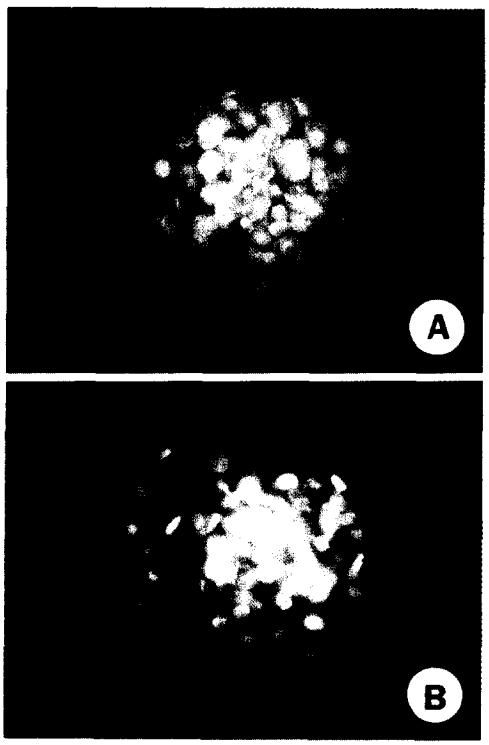

Fig. 2. Fluorescence micrographs of nuclei stained with bisbenzimide (A) or differentially stained with bisbenzimide and propidium iodide (B).
(A) Total cell number (TCN) of *in vitro* produced bovine expanded blastocyst at 8 day after IVF (TCN; 114). ×300. (B) ICM and TE cell numbers of *in vitro* produced bovine expanded blastocyst at 8 day after IVF. Blue ICM nuclei and pink TE nuclei are easily distinguished (ICM; 46, TE; 72). ×300.

Table 3. Numbers of ICM and TE cell of bovine blastocysts cultured in CR1 medium by using differential labelling

Blastocyst stage	No.	ICM cell number	TE cell number	Total cell number
Early	20	12.8± 5.9(6~27)[a]	30.5± 5.0(24~ 40)[a]	48.3± 7.8(32~ 57)[a]
Middle	21	26.3± 8.4(14~38)[b]	41.3± 8.2(30~ 58)[b]	68.5±12.7(54~ 88)[b]
Expanded	16	35.5±15.0(16~52)[b]	81.1±13.4(60~102)[c]	116.5±13.3(96~140)[c]

[a,b,c] Means in the same column without common superscripts are significantly different ($P<0.01$)

ing differential labelling with immunosurgery and polynucleotide-specific fluorochromes is summarized in Table 3. and Fig. 2B. As shown in Fig. 2B, the TE nuclei labelled with bisbenzimide and PI appeared red or pink and the ICM nuclei with bisbenzimide appeared blue. In Table 3, ICM cell numbers of the classified blastocysts to early, middle and expanded were 12.8±5.9, 26.3±8.4 and 35.5±15.0, respectively and TE cell numbers of the classified blastocysts to early, middle and expanded were 30.5±5.0, 41.3±8.2 and 81.1±13.4, respectively.

These results presented that ICM and TE cell numbers averaged two and three doublings between early and expanded blastocyst stage. Also, total cell number counted from ICM and TE nuclei by using differential labelling showed the increase pattern with development advance level and the results were similar to total cell number obtained from bisbenzimide treatment only (Table 2 and 3).

The differential labelling technique for ICM and TE nuclei in situ has two important advantages for the determination of cell numbers of ICM and TE and the analysis of regional interaction (Handyside and Hunter, 1984). The allocation of the cells to the ICM and TE of the blastocyst is fundamental importance for later development. Johnson and Ziomek (1981) noted that asynchrony of the compaction in 8-cell blastomeres is important for the subsequent generation of two types of cell lineage in the morula, which give rise to the ICM and TE of the blastocyst, respectively. Iwasaki *et al.* (1990) indicated that the cell-cell contacts of ICM cells in bovine blastocysts derived from *in vivo* fertilization were tighter than those from *in vitro* fertilization followed by culture *in vitro*, the proportion of ICM cells in blastocysts from *in vitro* fertilization decreased gradually with advance in development and the reduced cell proliferation of ICM cells of blastocyst cultured *in vitro* after *in vitro* fertilization than that of blastocysts cultured in rabbit oviducts after *in vitro* fertilization or blastocysts cultured *in vivo* may be the cause of the low pregnancy rates of blastocysts derived from *in vitro* fertilization. However, our results showed that the proportion of ICM cells was not decreased according to the development progression. Also, these results indicated that CR1 is a appropriate culture medium for bovine embryo development that not decreased ICM proportion with higher total cell number at expanded blastocyst stage when compared with the results obtained by Iwasaki *et al.* (1990).

Therefore, the differential labelling of ICM and TE nuclei in situ is a very useful technique to evaluate embryo qualities and can be used as an indicator on study of preimplantation embryo development.

Ⅳ. SUMMARY

The objective of this study was to examine

the cell number of Total, ICM and TE cells of bovine blastocysts according to development progression cultured in CR1 medium, which was reported as successfully supporting medium for preimplantaion bovine embryo development to the blastocyst stage, by differential labelling of the nuclei with immunosurgery and polynucleotide-specific fluorochromes. Blastocysts were obtained at day 8 after *in vitro* fertilization and classified to early, middle, expanded stage according to the developmental morphology; blastocoel expansion and zona thickness. Also, blastocysts in the same category were divided into two parts to check the Total cell number by using bisbenzimide only and ICM, TE and Total cell number by using immunosurgery and two polynucleotide-specific fluorochromes. 1) The development rate of blastocysts at day 8 after *in vitro* fertilization was 29.3% and classified blastocysts to early, middle, expanded and hatching stage were 8.7, 9.9, 7.6 and 3.1%, respectively. 2) The numbers of total blastomere using bisbenzimide in the classified blastocysts to early, middle and expanded were 46.9±8.6, 66.2±12.5 and 122.8±14.4, respectively. This indicated that CR1 is a appropriate culture medium for bovine embryo development. 3) The count of ICM and TE cell number by using differential labelling with immunosurgery and polynucleotide-specific fluorochromes in the classified blastocysts to early, middle and expanded ; ICM cell numbers of were 12.8±5.9, 26.3±8.4 and 35.5±15.0, respectively and TE cell numbers were 30.5±5.0, 41.3±8.2 and 81.1±13.4, respectively. These results presented that the increase of ICM and TE cell numbers averaged two and three doublings between early and expanded blastocyst stage and also total cell number counted from ICM nuclei and TE nuclei by using differential labelling showed the increase pattern with development advance level and the results were similar to total cell number obtained from bisbenzimide treatment only. Therefore, the differential labelling of ICM and TE nuclei in situ is a very useful technique to evaluate embryo qualities and can be used as an indicator on study of preimplantation embryo development.

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