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Early Development Bovine Zygotes Co-cultured with Cumulus Cells

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牛 體外受精卵의 初期發生에 미치는 卵丘細胞의 影響

박춘근 · 여인서 · 김정익
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적 요

소의 난포난자를 체외에서 성숙시켜, 수정후 8시간에서 난구세포가 부착된 난자와 기계적으로 완전히 제거한 난자를 10% FCS가 첨가된 TCM-199배양액에 옮겨, 수정 56시간에서 2~8세포기로 분할한 난자만을 5일간 계속 배양하여 초기배의 발육 상황을 검토하였다. 그 결과, 난구세포가 부착된 난자(31%)는 제거한 난자(15%)에 비해 상실배와 배반포기배까지의 발달이 유의적으로 높았다($p<0.01$). 한편, 난구세포와의 공동배양 조건하에서, 발생배지에 첨가하는 단백질원의 영향을 조사한 결과, BSA(59%)를 첨가한 경우 FCS(32%) 또는 CS(37%) 첨가보다 초기배의 발육율이 유의적으로 높았다($p<0.01$). 이상의 결과로부터, 소의 수정란과 난구세포와의 공동배양시 BSA가 초기발생에 촉진적 효과가 있음이 시사되었다.

Ⅰ. INTRODUCTION

One-cell bovine embryos cultured in a variety of conventional culture systems rarely cleave beyond 8- to 16-cells, whereas 16-cell embryos frequently develop to blastocysts(Wright and Bondioli, 1981; Camous et al., 1984). Such a developmental block of embryos *in vitro* have been observed in other species including pig(4-cell; Davis and Day, 1978), mice(2-cell; Goddard and Pratt, 1983) and hamster(2-cell; Farrel and Bavister, 1984). Although it is unknown why such the block does occur, one of the possible reasons is a result of suboptimal culture conditions. To solve this problem new culture system in which early embryos are cultured with other cells is recently developed. It has been shown that oviductal cells(Eyestone and First, 1989), fibroblasts(Gandolfi and Moor, 1987; Wiemer et al., 1987) and trophoblastic vesicles(Heymen et al., 1987) enhance the development of bovine embryos *in vitro*. The development of bovine embryos fertilized *in vitro* is also emhanced when they were co-cultured with cumulus monolayers(Goto et al., 1988, 1989). However, developmental capacity of the bovine embryos with cumulus cells on early cleavage and development were not determined.

The present study was to examine the effect of cumulus cells on the development of bovine embryos *in vitro*. It was also examined whether different protein sources supplemented to the culture medium could affect the development of bovine embryos co-cultured with cumulus cells.

Ⅱ. MATERIALS AND METHODS

1. Recovery of oocytes

Ovaries were removed immediately after

slaughter of cows at different stages of their reproductive cycle and transported to the laboratory in a saline solution with 100 μg penicillin G/ml and 100 μg streptomycin/ml maintained between 32 and 35℃. Follicular oocytes were aspirated from the follicles 3~5 mm in diameter by means of a 24-gage hypodermic needle attached to a 1-ml disposable syringe. The follicular contents were deposited on to a watchglass in which a little amount of a culture medium, TC-199 with Earle's salts buffered with 25mM-N-2-hydroxyethylpiperazine N-2-ethane sulfonic acid(Hepes) and supplemented with 10%(v/v) heat-inactivated fetal calf serum (FCS; Gibco, Life Technologies, Inc., USA), 100 μg penicillin G/ml and 100 μg streptomycin/ml has been previously presented. The cumulus-oocytes complexes were recovered under a streomicroscope and selected as described by McGaughey(1978), using only oocytes with evenly pigmented cytoplasm and completely surrounded by a dense layer of cumulus.

2. Culture of oocytes

Oocytes collected from the follicles were washed four times with TCM-199. The medium under paraffin oil was pre-warmed for at least 2~3 h at 39℃ in an atmosphere of 5% CO_2-95% air with high humidity to equilibrate the gas phase and the temperature. After wasing, about 10 cumulus-oocytes complexes were transfered into 100μl aliquots of medium covered with warm paraffin oil in a polystyrene culture dish. Oocytes were cultured for 22~24 h at 39℃ under an atmosphere of 5% CO_2-95% air with high humidity.

3. *In-vitro* fertilization

The basic medium for the manipulation of spermatozoa and fertilization of oocytes was essentially the same as that used by Brackett and Oliphant(1975) for the fertilization of rabbit eggs *in vitro* and designated as BO medium.

Frozen semen obtained from a Holstein bull was thawed in a water bath at 35~37℃ for 1 min. Spermatozoa were washed twice in a medium containing 10 mM caffeine-sodium benzoate(Sigma Chemical Co., St Louis, MO, USA) by centrifugation at 833 g for a period of 10 min each. The sperm pellet was resuspended in the same medium as used for washing to give a sperm concentration of $5~10\times10^6$/ml. A 50 μl sample of the sperm suspension was introduced into 50μl of the medium that contained bovine serum albumin(BSA; crystallized and lyophilized, essentially globulin free, No. A-7638; Sigma Chemical Co.), porcine intestinal mucosal heparin (176 USP unit/ml; Sigma Chemical Co.) and no caffeine. This medium had been previously covered with warm paraffin oil in a polystyrene culture dish. The mixture gave final concentrations of $2.5~5\times10^6$ spermatozoa/ml, 10 mg BSA/ml, 10 μl heparin/ml and 5mM-caffeine. They were incubated at 39℃ in an atmosphere of 5% CO_2-95% air with high humidity.

4. *In-vitro* developent

At 8 h after insemination, a portion of oocytes were freed from cumulus cells by repeated pipetting. The oocytes with cumulus cells and denuded ones were transferred into 0.1 ml drop of TC-199 medium with Earle's salts buffered with 25 mM-Hepes and supplemented with 10% FCS, 10% calf serum(CS; Gibco, Life Technologies, Inc., NY, USA) or 10 mg/ml BSA covered by paraffin oil. At 48 h after culture(56 h after insemination) the oocytes were examined under a dissecting microscope and non-cleaved oocytes were removed. The embryos were further incubated under 5% CO_2 in

air at 39℃ for 5 days. Medium was changed every two days during the culture.

Ⅲ. RESULTS

The embryos cleaved to 2- to 8-cell stage 56h after insemination were cultured further for 5 days in the medium 10% FCS. Table 1 shows that proportions of embryos developed to morula or blastocyst stage significantly higher ($P<0.01$) in those cultured with cumulus cells(31%) than without cumulus cells(15%).

As shown in Tabel 2, when the embryos were cultured with cumulus cells in the medium with different protein sources, the highest proportion(59%) of embryos developed to morula or blastocyst stage was obtained in the medium with BSA ($P<0.001$). Higher proportions of embryos were degenerated during culture with FCS(15%) or CS(14%) compared with BSA (3%).

Ⅳ. DISCUSSION

The beneficial effect of cumulus cells on maturation of oocytes *in vitro* has been reported for human(Kennedy and Donahue, 1969), rabbit and cow(Robertson and Baker, 1969) and mouse(Cross and Brinster, 1970). It has also been reported that cumulus cells support normal fertilization of eggs and further development of embryos in rabbit(Motilik and Fulka, 1981), pig(Fulka and Motlik, 1980.) and sheep (Staigmiller and Moor, 1984). Several workers (Critser et al., 1986; Lu et al., 1987, 1988; Lutterbach et al., 1987; Xu et al., 1987; Fukui

Table 1. Development of bovine embryos cultured with or without cumulus cells

Cumulus cell	No. of embryos used*	No. of embryos developed (%)		
		2- to 16-cell	Morula or blastocyst	Degenerated
+	128	78(61)	25+15**(31)[a]	10(8)
−	130	90(69)	14+ 6 (15)[b]	20(15)

* The embryos cleaved to 2- to 8-cell stage 56h after insemination were further cultured for 5 days.

** The first figure denotes the number of embryos at morular stage and the second one denotes the number of embryos at blastocyst stage.

a, b: $P<0.01$ (x^2-test).

Table 2. Develpment of bovine embryos cultured with cumulus cells in the presence of different protein sources.

Proteins	No. of embryos used*	No. of embryos developed (%)		
		2- to 16-cell	Morula or blastocyst	Degenerated
FCS	138	72(52)	28+17**(32)[a]	21(15)
CS	148	72(49)	31+25 (37)[a]	20(14)
BSA	145	56(38)	47+38 (59)[b]	4(3)

* The embryos cleaved to 2- to 8-cell stage 56h after insemination were further cultured for 5 days.

** The first figrue denotes the number of embryos at morula stage and the second one denotes the number of embryos at blastocyst stage.

a, b: $P<0.001$ (x^2-test).

and Ono, 1988, 1989) have used cumulus cells to induce nuclear and cytoplasmic matiration of bovine oocytes, and suggest that these cells enhance the fertilization and subsequent development of the matured oocytes.

In the present study, the development of early bovine embryos was enhanced when they were cultured with cumulus cells. Although, at present, the mechanism of cumulus cells to support development of oocytes is not clear, one of the possible explanations is that some factors secreted from cumulus cells may stimulate oocyte development. If this is true, the development of oocytes cultured with cumulus cells without cell-contact may be facilitated. However this is not necessarily demonstrated, and some investigators suggested that contact between cumulus cells and oocytes is most important factors. Although the growth of cumulus cells in medium with BSA was not so superior as in FCS or CS, it seems that the early development of bovine embryos co-cultured with cumulus cells after *in vitro* fertilization is maintained most efficiently by BSA.

Leibfried-Rutledge et al.(1986) demonstrated that FCS seems to be superior for *in-vitro* maturation of cumulus oocytes complexes in hamster and cow. On the other hand, Kane(1983) has reported that BSA can support cell multiplication and hatching of rabbit embryos in culture but to efficacy may be different according to the commercial lots. Since BSA contains less unknown factors than FCS and CS, the results obtained in the present study may suggest that it is much easier to analyse factors necessary for early development of oocytes *in vitro*.

V. SUMMARY

Bovine follicular oocytes were matured, fertilized and cultured *in vitro*. Oocytes with or without cumulus cells were transferred into TC-199 medium supplemented with 10% FCS 8h after insemination, and embryos cleaved to 2- to 8-cell stage 56h after insemination were cultured further for 5 days. The proportions of embryos developed to morular and blastocyst stages were significantly higher ($P<0.01$) in those cultured with(31%) than without(15%) cumulus cells. When the embryos were cultured with cumulus cells in the medium with different protein sources, the highest proportion(59%) of embryos developed to morular or blastocyst stage was obtained in medium with BSA($P<0.001$). Higher proportions of embryos were degenerated during culture with FCS(15%) or CS(14%) compared with BSA(3%). The present results indicate the early development of *in-vitro* fertilized bovine embryos can be maintained efficiently by BSA when they were co-cultured with cumulus cells.

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