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Effect of Different Protein Concentrations on the Development of Bovine Zygotes Co-cultured with Cumulus Cells

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卵丘細胞와의 共同培養時 牛體外受精卵의 初期發生에 미치는 蛋白質 濃度의 影響

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적 요

소의 체외수정란과 난구세포와의 공동배양시 발생배지에 FCS, CS 또는 BSA를 여러 농도로 첨가해 상실배 또는 배반포기배까지의 발생율을 비교 검토했다. 그 결과, 단백질원을 전혀 첨가하지 않은 경우 발생율이 11%로 첨가의 경우에 비해 낮았다.

한편, 1~10% FCS(27~38%), 1~10% CS(29~35%) 및 1~10mg/ml BSA(57~59%)를 첨가한 경우, 첨가단백질 농도 사이에서 발생율에 유의차는 인정되지 않았으나, 20%의 FCS와 CS, 20mg/ml의 BSA농도에서 보다 높은 발생율을 보였다($P<0.05$). 첨가 단백질 종류로만 발생율을 비교한 경우, FCS(17~35%) 및 CS(9~35%) 첨가에 비해 BSA(35~59%) 첨가시 높은 발생율이 인정되었다. 한편, BSA가 첨가된 배지에서 난구세포의 증식은 관찰되지 않았는데, 이와 같은 결과로부터 초기배의 발육과 난구세포의 성장 사이에는 상관관계가 없는 것으로 추측되었다.

I. INTRODUCTION

The increased viability of certain cells cultured in the presence of a feeder layer of another cell types has been documented in numberous *in-vitro* culture systems(Kohler and Milstein, 1975; Martin, 1981). Enhanced *in-vitro* development of embryos cultured together with irradiated HeLa cells was first reported in the mouse(Cole and Paul, 1965).

Recently it has been shown that oviductal cells(Fukui et al., 1989; 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 enhanced when they were co-cultured with cumulus monolayers(Goto et al., 1988, 1989).

In previously our study(Park et al., 1992), it was examined the effect of protein sources supplemented to the culture medium on the development of oocytes with cumulus cells.

Since, however, only single concentration (10% for FCS and CS and 10mg/ml for BSA) of protein sources was used in the study, the purpose of the present study was examine the effect of different concentrations of each protein source on the development of bovine embryos with cumulus cells.

Ⅱ. MATERIALS AND METHODS

1. Collection of oocytes

Bovine ovaries were removed immediately after slaughter 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 25 mM-N-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 cells.

2. Culture of oocytes for maturation

Oocytes collected from the follicles were washed four times with TC-199 medium. The medium under paraffin oil was pre-warmed for 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 washing, about 10 cumulus-oocytes complexes were transferred into 100 μl aliquots of medium covered with paraffin oil in a polystyrene culture dish(35×10 mm, Termo, Tokyo, Japan). 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 composition of the basic medium used for the treatment of spermatozoa and the 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. The medium was sterilized prior to use by means of a millipore filter with pore size of 0.22 μm.

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 $5\sim10\times10^6$/ml. A 50 μl sample of the sperm suspension was introduced into 50 μl of the medium that contained 20 mg bovine serum albumin(BSA; crystallized and lyophilized, essentially globulin free, NO. A-7638; Sigma Chemical Co.)/ml, 20 μg porcine intestinal mucosal heparin(176 USP unit/ml; Sigma Chemical Co.)/ml and no caffeine.

This medium was prepared in the center of a culture dish and covered with paraffin oil. The dished were kept in a CO_2 incubator(5% CO_2 in air at 39℃) for about 2~3 h for equilibration with the gas phase and temperature. The mixture gave final concentrations of $2.5\sim5\times10^6$ spermatozoa/ml, 10 mg BSA/ml, 10 μg heparin/ml and 5 mM-caffeine. They were incubated at 39℃ in an atmosphere of 5% CO_2-95% air with high humidity.

4. Co-culture of zygotes with cumulus cells

At 8 h after insemination, oocytes with cumulus cells were transfered into 0.1 ml drop of TC-199 medium with Earle's salts buffered with

25 mM-Hepes and supplemented with FCS, CS (0, 1, 5, 10 or 20%) or BSA(0, 1, 5, 10 or 20 mg/ml) covered by paraffin oil after washing more than twice with the same medium. Only cleaved oocytes 48 h later were further incubated for 5 days under 5% CO_2 in air at 39℃ examined for their developmental stages. Medium was changed every two days during the culture.

Ⅲ. RESULTS

As shown in Table 1, when the culture medium was supplemented with various concentrations of FCS, the higher proportions(27~38%) of oocytes developed to morular or blastocyst stage were obtained at the concentrations of 1~10% than 0 or 20%(11~17%). On the other hand, the highest proportion(15%) of degenerated embryos were obtained with 20% FCS. The growth of cumulus cells was better in 20% than 0, 1, 5 or 10% FCS during co-culture.

Table 2 shows that there is no difference in the proportions of oocytes developed to morular or blastocyst stage in the medium with CS at the concentrations of 1, 5 and 10%. However,

Table 1. Effect of different concentrations of fetal calf serum(FCS) in the medium on development of oocytes with cumulus cells

Concentration of FCS (%)	No. of embryos used*	No. of embryos developed (%)		
		2- to 16-cell	Morula and blastocyst	Degenerated
0	53	41(77)	5 + 1** (11)[a]	6(11)
1	51	34(67)	7 + 7 (27)[ab]	3(6)
5	66	37(56)	10+15 (38)[b]	4(6)
10	48	29(60)	10+ 6 (33)[ab]	3(6)
20	48	33(69)	5 + 3 (17)[a]	7(15)

* The embryos cleaved to 2- to 8-cell stage 56 h 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 : $P<0.05$(χ^2-test)

Table 2. Effect of different concentrations of calf serum(FCS) in the medium on development of oocytes with cumulus cells

Concentration of CS (%)	No. of embryos used*	No. of embryos developed (%)		
		2- to 16-cell	Morula and blastocyst	Degenerated
0	53	41(77)	5 + 1** (11)[a]	6(11)
1	59	38(64)	10+ 7 (29)[ab]	4(7)
5	57	34(60)	12+ 8 (35)[b]	3(5)
10	44	26(59)	10+ 4 (32)[ab]	4(9)
20	45	34(76)	3 + 1 (9)[a]	7(16)

* The embryos cleaved to 2- to 8-cell stage 56 h 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 : $P<0.05$(χ^2-test)

Table 3. Effect of different concentrations of bovine serum albumin(BSA) in the medium on development of oocytes with cumulus cells

Concentration of BSA (%)	No. of embryos used*	No. of embryos developed (%)		
		2- to 16-cell	Morula and blastocyst	Degenerated
0	53	41(77)	5 + 1** (11)[a]	6(11)
1	70	27(39)	15+26 (59)[b]	2(3)
5	68	27(40)	16+23 (57)[b]	2(3)
10	69	29(42)	19+20 (57)[b]	1(1)
20	68	38(56)	19+ 9 (35)[c]	6(9)

* 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 : $P<0.05$ at least (χ^2-test)

the proportion significantly decreased at 0 and 20% CS($P<0.05$). A higher proportion(11 and 16%) of embryos degenerated was also obtained in medium with 0 and 20% CS.

When the medium supplement with BSA was used(Table 3), the proportions of oocytes developed to morular or blastocyst stage were higherin the concnetrations of 1~10mg/ml (57~59%) than 0 or 20mg/ml(11~35%, $P<0.05$ at least). Although the developmental capacity of embryos was improved by BSA compared with FCS and CS, growth of cumulus cells was not so remarkable, especially in the concentration of 1mg BSA/ml at which only partial growth of cumulus cells was observed.

Ⅳ. DISCUSSION

It was clarified in our study previously(Park et al., 1992) that culture medium containing BSA was effective in supporting development of 2- to 8-cell embryos to morular and blastocyst stage *in-vitro*. This results was confirmed in the present study. However, growth of cumulus cells was very poor in the medium supplemented with BSA, in contrast to that with FCS or CS. Leibfried-Rutledge et al.(1986) reported that BSA at the doses tested was not capable of maintaining the viability of cumulus cells and this was more apparent in the cow than in the hamster.

Although the cumulus expansion induced by serum is also reported in mouse(Eppig, 1979, 1980), it is clear from the present results that growth of cumulus cells does not necessarily support the development of embryos. Ogawa and Mars(1987) reported that more than 90% of 2-cell mouse embryos developed to the blastocyst both in protein-free medium embryos and medium supplemented with either FCS or BSA, but the proportion of hatching blastocysts was significantly decreased in the protein-free medium.

Rexroad and Powell(1988) reported that Ham's F-10 with FCS was not satisfactory for the development of sheep embryos. In the present study, the culture medium with 5% FCS or CS resulted in increased embryo development, whereas the medium was ineffective for the growth of cumulus cells. The indicates again that there is no correlation between cumulus expansion and oocytes development.

Although the optimal culture conditions for co-culture of early bovine embryos has not been

identified, Gandolfi and Moor(1988) have suggested that *in-vitro* secretory profile of cultured oviductal cells was similar to that in *in-vitro* condition and they proposed that the oviductal cells may secrete a mitogen that stimulates embryonic development. If the cumulus cells secrete such substance, BSA may support the function of the cells and thus stimulate the development of bovine embryos beyond 8-cell stage.

V. SUMMARY

Fetal calf serum(FCS), calf serum(CS) and bovine serum albumin(BSA) were evaluated as protein sources supplemented to the culture medium for *in-vitro* development of bovine embryos with cumulus cells. High concentration of FCS and CS(each 20%) and BSA(20 mg/ml) did not support development of the embryos. The proportions of embryos developed were not significantly different amoung concentrations of 1~10% for FCS(27~38%) and CS(29~35%), and those of 1~10 mg/ml for BSA(57~59%). Although higher proportions of embryos developed were obtained with BSA(35~59%) than FCS(17~38%) and CS(9~35%), the growth of cumulus cells during culture was much inhibited in the medium with BSA. This result indicates that there is no correlation between cumulus expansion and early development of embryos.

VI. REFERENCES

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