CO-CULTURE OF BOVINE EMBRYOS WITH CUMULUS CELLS

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Summary

Bovine embryos/ova obtained from *in-vitro* fertilization were either co-cultured on a monolayer of bovine cumulus cells or cultured in medium alone. Embryos/ova co-cultured with cumulus cells developed to 8-cell (30.9%), morula (29.8%) and blastocyst stages (26.6%) after 3-4, 5-6, and 7-8 days of culture, respectively, while embryos/ova cultured in medium alone failed to develop beyond 8-cell (0-13.3%), morula (0-1.5%) and blastocyst stages (0%).

The results of this study demonstrated the beneficial effect of cumulus cells on the development of bovine embryos.

(Key Words: *In-Vitro* Fertilization, Embryo, Bovine, Cumulus Cells, Co-Culture)

Introduction

The requirements for development of early embryos of domestic animals have not been defined (Rexroad, 1989). Co-culture of embryos on monolayers of cells from the reproductive tract improved the development of embryos and may be a valuable tool for identification of interactions between the embryo and reproductive tract cells that play a specific role in the early development of embryos Rexroad, 1989).

Early bovine embryos benefit from co-culture Leibfried-Rutledge et al., 1989). Kuzan and Wright (1982) found that co-culture of bovine morulae in Minimal Essential Medium supplemented with fetal calf serum on either bovine uterine fibroblasts or bovine testicular fibroblasts resulted in more morulae developing to blastocysts and hatching than those cultured in medium alone. Voekle et al. (1985) demonstrated that bovine demi-embryos had much greater viability after 72 h co-culture on a monolayer than after 72 h culture in medium alone. Wiemer et al. (1987) demonstrated the same benefit of co-culture for intact embryos. Eyestone et al. (1987) co-cultured early bovine embryos (5 to 8 cells) on oviductal cell monolayers and found that co-cultured embryos developed to the late morula or blastocyst stage while embryos cultured in medium alone fail to develop beyond 16-cells.

Goto et al. (1988a) reported a birth of a female calf born to a recipient that had received blastocysts obtained from totally a *in-vitro* system utilizing a monolayer of cumulus cells. Normal calves were born from the transfers of the frozen-thawed blastocysts obtained from this *in-vitro* technique (Goto et al., 1988b). Furthermore, pregnancy was obtained from the transfer of bisected bovine blastocysts derived from totally *in-vitro* technique (Goto et al., 1988b). Similarly Lu et al. (1988) obtained calves from *in-vitro* techniques utilizing bovine oviduct cells.

In spite of these successful results the role of the monolayer in a co-culture system is not clear. The purpose of this study was to verify further the importance of co-culture system utilizing bovine cumulus cell.

Materials and Methods

Collection and culture of oocytes

Ovaries were collected from cows at a local slaughter house and brought to the laboratory. The precise details of collection and culture of cumulus-oocyte complexes have been described previously (Goto et al., 1988a).

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Sperm preparation

Frozen semen samples of epididymal origin were prepared according to our previous report (Goto et al., 1989). The plastic straws of epididymal semen were thawed in warm water (32-35°C) and then washed 3 times (centrifugation at 700 g for 5 to 8 min) with BO (Brackett and Oliphant, 1975) medium without bovine serum albumin but supplemented with 5 mM caffeine. The spermatozoa were then preincubated for 3 h in 5 % CO_2 in air at 38.5°C as a 110 μl microdrop (12.5×10^6 sperm/ml) in BO medium containing bovine serum albumin (5 mg/ml: Sigma, A-4387, St. Louis, MO, U.S.A.) and caffeine (2.5 mM) before insemination. Microdrops were covered with liquid paraffin.

Insemination and subsequent culture

After incubation for 20-24 h the cumulus-oocyte complexes were inseminated by transfer to the sperm microdrop (about 15 oocytes/microdrop). After 6 h of insemination, the ova with cumulus cells were transferred into the development medium (25 mM-Hepes TCM 199 with Earle's salt; Gibco, Cat.380-2340, NY, U.S.A.+ 5 % neonatal calf serum (NCS); Gibco, Lot. 22P-4457, Experiment 1; 25 mM-Hepes TCM 199 + 1 % NCS + 1 mM sodium pyruvate, Experiment 2) and cultured for further development in a CO_2 incubator at 38.5°C. The culture medium (2.5 ml) in a polystyrene dish (35 M/M, Terumo, Tokyo, Japan) was covered with liquid paraffin.

Experiment 1

Twenty-four, 48, 72 or 96 h after insemination, cumulus cells attached to the ova were removed by pipetting, leaving a monolayer attached to the bottom of the culture dish. The ova were then cultured on the cumulus cell monolayer in the dish for 6-7 days. The incubation medium was replaced with new medium every 24-48 h. All of the culture media used were supplemented with antibiotics (100 i.u. penicillin/ml + 100 μg streptomycin/ml). The embryos were examined under microscope every 24 h after the removal of attached cumulus cells.

Experiment 2

Eight, 48 or 72 h after insemination, cumulus cells attached to the ova were removed by pipetting and the ova were transferred into the new culture dished containing new medium (nonco-culture groups). In the co-culture group, cumulus cells attached to the ova were removed by pipeting 72 h after insemination and the culture medium was replaced with new medium. The subsequent method was the same as that of exp. 1.

Development rates

Ova were examined for degree of development every 24 h and the Chi square test was used for statistical comparison of differences between groups.

Results

The effect of the removal of cumulus cells surrounding embryos on *in-vitro* development of bovine embryos is shown in table 1. The only

TABLE 1. EFFECT OF THE REMOVAL OF CUMULUS CELLS SURROUNDING EMBRYOS ON *IN VITRO* DEVELOPMENT OF BOVINE OOCYTES FERTILIZED *IN VITRO*

Group	Removal[1] of cumulus cells surrounding embryo[2]	No. of oocytes used	% of oocytes developed to:		
			8-cell ≦ (3-4)[3]	Morula ≦ (5-6)	Blastocyst ≦ (7-8)
1	24	184	28.3[a]	21.7	12.0
2	48	171	28.7[a]	22.2	11.1
3	72	135	26.7[a]	25.2	13.3
4	96	170	17.1[b]	16.5	11.2

Within columns, means with no superscipt in common are different ($p < 0.05$).

[1] Hours after insemination.

[2] After removal of cumulus cells surrounding embryos, embryos were co-cultured with cumulus cells attached to the bottom of culture dish.

[3] Days after insemination.

developmental difference between groups was that ova treated 96 h after insemination developed into less 8-cell embryos ($p < 0.05$) than in the case of earlier treatments. There were no significant differences among the 4 groups in ova that progressed to the blastocyst stages.

The effect of the co-culture of cumulus cells on *in-vitro* development of bovine embryos is shown in table 2. Co-culture of embryos with cumulus cell layer (group 4) significantly increased the numbers of embryos which developed into 8-cell, morula and blastocyst stages ($p < 0.01$). Culturing of embryos in medium alone did not support the development of embryos beyond blastocyst stage regardless of the time of the removal of cumulus cells surrounding embryos.

Discussion

The result of this study clearly demonstrated that early bovine embryos benefit from co-culture with cumulus cells. Similarly, several investigators (Kajihara et al., 1987; Eyestone et al., 1987; Goto et al., 1988a; Fukui and Ono, 1988; Lu et al., 1988) reported that co-culture of bovine embryos with reproductive cells is beneficial. The role of the monolayer in a co-culture system is not clear. A number of possibilities have been suggested, including provision of required metabolites, specific growth stimulators, and detoxification of medium (Rexroad, 1989).

Eyestone and First (1988) reported that conditioned medium from an oviductal cell culture stimulated the development of early bovine embryos. In contrast, Allen and Wright (1984) found that conditioned medium was not an adequate substitute for the cellular monolayer for the development of early porcine embryos. Rexroad and Powell (1986; 1988) also observed that conditioned medium did not substitute for co-culture in the development of early ovine embryos. These observations suggest that physical contact between the embryos and the monolayer may be necessary for expression of the co-culture effect. Allen and Wright (1984) proposed that membrane extensions through the zona pellucida might provide direct contact between the monolayer and developing embryos, but such connections have not been demonstrated in culture (Rexroad, 1989). Although expansion of the beneficial effect of the co-culture system on embryonic development *in-vitro* remains to be clarified, the present results confirm that the system is of value for *in-vitro* culture of embryos.

TABLE 2. EFFECT OF THE CO-CULTURE OF CUMULUS CELLS ON *IN VITRO* DEVELOPMENT OF BOVINE OOCYTES FERTILIZED *IN VITRO*

Group	Removal[1] of cumulus cells surrounding embryo	No. of oocytes used	% of oocytes developed to:			
			4-cell ≦ (3)[2]	8-cell ≦ (3-4)	Morula ≦ (5-6)	Blastocyst ≦ (7-8)
1[3]	8	85	5.9[a]	0[a]	0[a]	0[a]
2[3]	48	156	28.8[b]	8.7[b]	1.5[a]	0[a]
3[3]	72	98	30.6[bc]	13.3[b]	1.0[a]	0[a]
4 Co-culture[4]	72	94	47.9[c]	30.9[c]	29.8[b]	26.6[b]

Within columns, means with no superscript in common are different ($p < 0.01$).
[1] Hours after insemination.
[2] Days after insemination.
[3] After removal of cumulus cells surrounding embryos, embryos were cultured in medium alone.
[4] After removal of cumulus cells surrounding embryos, embryos were co-cultured with cumulus cells attached to the bottom of culture dish.

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Literature Cited

Allen, R.L. and R.W. Jr. Wright. 1984. *In vitro* develop-

ment of porcine embryos in coculture with endometrial cell monolayers or culture supernatants. J. Anim. Sci. 59:1657-1661.

Brackett, B.G. and G. Oliphant. 1975. Capacitation of rabbit spermatozoa in vitro. Biol. Reprod. 12:260-274.

Eyestone, W.H. and N.L. First. 1988. Co-culture of bovine embryos with oviductal tissue. In: Brief communication: 11th International Congress on Animal Reproduction and Artificial Insemination. pp.471-473.

Eyestone, W.H., J. Vignieri and N.L. First. 1987. Co-culture of early bovine embryos with oviductal epithelium. Theriogenology (Abs.) 27:228

Fukui, Y. and H. Ono. 1988. *In vitro* development to blastocyst of *in vitro* matured and fertilized bovine oocytes. Vet. Rec. 122:282.

Goto, K., Y. Kajihara, S. Kosaka, M. Koba, Y. Nakanishi and K. Ogawa. 1988a. Pregnancies after co culture of cumulus cells with bovine embryos derived from *in-vitro* fertilization of *in-vitro* matured follicular oocytes. J. Reprod. Fert. 83:753-758.

Goto, K., Y. Kajihara, M. Koba, Y. Nakanishi and K. Ogawa. 1988b. Pregnancy in cattle after transfer of bisected blastocysts obtained from *in-vitro* fetilization of oocytes matured *in-vitro*. Asian-Australasian J. Anim. Sci. 1:153-156.

Goto, K., Y. Kajihara, M. Koba, S. Kosaka, Y. Nakanishi and K. Ogawa. 1989. *In vitro* fertilization and development of *in vitro* matured bovine follicular oocytes. J. Anim. Sci. 67:2181-2185.

Kajihara, Y., K. Goto, S. Kosaka, Y. Nakanishi and K. Ogawa. 1987. *In vitro* fertilization of bovine follicular oocytes and their development up to hatched blastocysts *in vitro*. Jpn. J. Anim. Reprod. 33:173-180.

Kuzan, F.B. and R.W. Jr. Wright. 1982. Blastocyst expansion, hatching, and attachment of porcine embryos co-cultured with bovine fibroblasts *in vitro*. Anim. Reprod. Sci. 5:57-63.

Leibfried-Rutledge, M.L., E.S. Critser, J.J. Parrish and N.L. First. 1989. *In vitro* maturation and fertilization of bovine oocytes. Theriogenology 31:61-74.

Lu, K.H., I. Gordon, H.B. Chen, M. Gallagher and H. McGovern. 1988. Birth of twins after transfer of cattle embryos produced by *in vitro* techniques. Vet. Rec. 122:539-540

Rexroad, C.E. Jr. 1989. Co-culture of domestic animal embryos. Theriogenology 31:105-114.

Rexroad, C.E. Jr. and A.M. Powell. 1986. Co-culture of sheep ova and cells from sheep oviduct vesicles. Theriogenology (Abs.) 25:187.

Rexroad, C.E. Jr and A.M. Powell. 1988. Co-culture of ovine ova with oviductal cells in medium 199. J. Anim. Sci. 66:947-953.

Voekle, S.A., G.F. Amborski, K.G. Hill and R.A. Godke. 1985. Use of uterine-cell monolayer culture system for micromanipulated bovine embryos. Theriogenology 24:271-281.

Wiemer, K.E., R.S. Denniston, G.F. Amborski, K.L. White and R.A. Godke. 1987. A fetal fibroblast monolayer system of *in vitro* culture of bovine embryos. J. Anim. Sci. 65(Suppl. 1): 122(Abs.).