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NONSPECIES-SPECIFIC EFFECTS OF MOUSE OVIDUCTS ON THE DEVELOPMENT OF BOVINE IVM/IVF EMBRYOS BY A SERUM FREE CO-CULTURE

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ABSTRACT

In Experiment 1, development of bovine embryos derived from in vitro-matured (IVM) and in vitro-fertilized (IVF) oocytes was examined under 4 culture conditions: 1) co-culture with mouse ampullae continuously for 8 d, 2) co-culture with mouse ampullae that were replaced with fresh ampullae at 48-h intervals, 3) co-culture with bovine granulosa cell monolayers, and 4) culture in medium alone. Culture medium consisted of tissue culture medium 199 (TCM-199) supplemented with 1% fetal calf serum (FCS). Inseminated oocytes were transferred to each of the culture treatment 24 h after insemination and were cultured for 8 d. The number of blastocysts per number of cleaved ova obtained after co-culture with mouse ampullae (42.9%) was significantly ($P<0.05$) higher than that obtained after co-culture with granulosa cell monolayers (28.3%) or culture without cells (4.2%). In Experiment 2, the developmental ability of bovine IVM/IVF embryos co-cultured with mouse ampullae supplemented with or without serum was examined. When serum was excluded from the culture medium, 26.4% (33/125) of the total number of embryos cultured were able to develop to the blastocysts stage using this co-culture system. This value was comparable to that obtained in a serum-supplemented co-culture system (30.7%; 39/125). In addition, the developmental ability of embryos that reached to the 4-cell stage or beyond at 46 to 48 h after insemination was not significantly different when the embryos were co-cultured with mouse ampullae with (38.5 vs 44.6%) or without (37.0 vs 33.8%) serum.

Key word: bovine embryo, co-culture, mouse oviduct

INTRODUCTION

The first successful co-culture of embryos was reported in mice using organ culture system of fallopian tubes (2). Recently, intraspecies-specific effects of oviduct on the

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development of embryos in vitro were demonstrated in several species using a co-culture system (9,14,17,26,27). Isolated mouse ampullae maintained in organ culture have been reported to have interspecies-specific effects on the development of hamster (25) and porcine (22,23) embryos. Interspecies-specific effects of oviduct on the development of bovine embryos have been reported in rabbit oviducts in vivo (24). In contrast, Sparks et al. (32) found that bovine 1-cell embryos fertilized in vivo could not develop to morula/blastocyst stage in explanted mouse oviducts maintained in organ culture. The development of early bovine embryos in vitro is usually blocked before or at 8- to 16-cell stage in culture medium supplemented only with serum (6,8,35). Although, this developmental block can be overcome by culturing embryos in a medium containing cumulus cells or oviduct cell monolayers, or in media conditioned by these cells (1,7,12,13,15,21,28,31,36), these culture systems did require serum supplementation. Recently, it has been demonstrated that IVM/IVF oocytes can develop in vitro to the morula stage in chemically defined medium without supplementation of serum and, alternatively, that serum factors are needed for blastocyst development (30).

In this study experiments were designed to examine 1) the nonspecies-specific effects of mouse ampullae on the development of bovine IVM/IVF embryos and 2) the effects of serum deletion from the culture medium with or without ampullae. In both experiments, in vitro maturation was completed without serum supplementation.

MATERIALS AND METHODS

Oocyte Recovery and Maturation

Ovaries were collected at a local abattoir and transported in a thermos flask at 35 to 39°C in 0.85% saline. Immature bovine oocyte-cumulus complexes (OCCs) were aspirated from small antral follicles (2 to 5 mm) using 18-gauge needle connected to 10-ml syringe within 3-4 h after ovary collection. Aspirated follicular contents were pooled in a 50-ml conical tube maintained at 39 °C for about 20 min. After collection of follicular contents, the sediment was recovered and transferred into m-Tyrode's solution (BO solution;4) supplemented with 1 mg/ml bovine serum albumin (BSA; lyophilized powder; Sigma Chemical Co. St. Louis, MO, USA; A7638). Only oocytes with an intact, compact cumulus oophorus and evenly granulated cytoplasm were selected under a stereomicroscope, using a fine pasteur pipette. Selected OCCs were washed once with BO solution and twice with maturation medium. After washing, 30 to 40 OCCs were placed in a 200-μl drop of maturation medium, drop covered with paraffin oil in 60 × 15-mm petri dishes (Nunclon, Nunc, Roskilde, Denmark), and cultured for 24 h for IVM. To exclude the effects of serum and BSA on oocyte maturation, a serum- and BSA-free maturation system was used (20). Maturation medium consisted of TCM-199 with modified Earle's salts (Nissui Pharmaceutical Co., Ltd., Japan) supplemented with 0.5 mM sodium pyruvate (Sigma; P2256), 3 mg/ml polyvinylpyrrolidone (PVP: M.W. 40,000; Nacalai Tesque Inc., Japan), 10 IU/ml hCG (Teikoku Hormone M.F.G. Co. Ltd., Japan), 1 μg/ml estradiol (Sigma; E8875), and 50 units/ml penicillin G (Sigma). All media were sterilized by 0.3-μm membrane filter (Toyo Roshi Kaisha, Ltd, Japan) and

stored at 4 °C. The oocytes were cultured under 5% CO_2 in air with 100% humidity at 39 °C.

In Vitro Fertilization

Spermatozoa for in vitro fertilization were prepared as described previously (34). Frozen semen from 1 bull was thawed at 39 °C for 10 sec, and 1 ml of the semen was layered on each 2 ml of 30 to 45% discontinuous percoll (Pharmacia Co., Ltd., Uppsala Sweden) gradient solution diluted with BO solution without glucose (29). After centrifugation of semen (500 × g, 10 min), the concentration of a final sperm pellet was counted with a hemocytometer. The medium used for IVF was BO solution without glucose supplemented with 3 mg/ml BSA and 10 μ g/ml heparin (sodium salt; Sigma; H3125). The sperm pellet was diluted with BO solution, as described above, at a concentration of 3 × 10^8 sperm/ml and preincubated for 1 h under 5% CO_2 in air with 100% humidity at 39 °C. At the end of the maturation period, 30 to 40 OCCs were transferred to a 200 μ l of IVF medium under paraffin oil, then the preincubated spermatozoa were introduced into the IVF medium at a final concentration of 2.4 × 10^6 sperm/ml. Incubation conditions for IVF were 5% CO_2 in air with 100% humidity at 39 °C for 22 to 24 h. After IVF, some oocytes were randomly collected from each replicate of Experiment 1 and then fixed in ethanol-acetic acid (3:1, v/v) and stained with aceto-orcein for assessment of fertilization (18). The fertilization rate of the oocytes was 80%; however, well-timed fertilization at 22 to 24 h after insemination was observed in 28 oocytes (70%) with male and female pronuclei or that developed just before the first cleavage stage, and polyspermic fertilization was also observed in 8% of inseminated oocytes (data not shown). From this result, approximately 90% of well-timed fertilized oocytes were estimated to have completed the first cleavage.

Co-culture of IVM/IVF Oocytes

Preparation of granulosa cells : At the time of oocyte collection, recovered granulosa cells from follicles were introduced into 2 ml of TCM-199 containing 0.2% hyaluronidase (Sigma; H3506), without BSA, maintained at 39 °C. After a few minutes, the cells were centrifuged for 5 min at 100 × g. Pelleted granulose cells were resuspended with TCM-199 containing 0.5 mM sodium pyruvate and 50 units penicillin G supplemented with 1% heat-treated FCS (Gibco Laboratories, Inc., Grand Island, NY, USA). The cells were introduced into 200-μ l drops of culture medium at a final concentration of 1 × 10^6 cells/ml, and then cultured in a CO_2 incubator for 44 h to form a granulosa cell monolayers.

Preparation of mouse ampullae : Mouse oviducts were recovered from 5- to 7-wk-old ICR mice superovulated with PMSG and hCG. The oviducts for co-culture were isolated 16 h after hCG treatment, and the isthmic and fimbrial regions of the oviducts were removed and the ampullae were longitudinally dissected to open the ducts (27). After several washings with each culture medium (TCM-199 + 1% FCS in Experiment 1 or TCM-199 + 0.1% PVP in Experiment 2, respectively), 2 dissected ampullae from

different mice were introduced into 200-μl drop of each culture medium.

After the IVF period, oocytes were stripped of cumulus cells by passing them through a fine-drawn pasteur pipette in IVF medium. After removal of cumulus cells, the oocytes were washed 2 times in culture medium. Oocytes were pooled and assigned randomly among the 4 treatments with 1 replicate.

In both experiments, 170 μl of the culture medium was removed from each culture drop and replaced with the same volume of fresh medium at intervals of 48 h under 5% CO_2 in air with 100% humidity at 39 °C. The oocytes were evaluated for cleavage at 46 to 48 h later and for development to the blastocyst stage at 7, 8 and 9 days after insemination.

Experiment 1

To examine the effects of mouse ampullae on the development of bovine IVM/IVF embryos to the blastocyst stage, bovine embryos were co-cultured with mouse ampullae. Co-cultures were performed over 8 d either with the same ampullae or with fresh ampullae replaced at 48-h intervals. The effects were confirmed by comparing the developmental rates of the embryos co-cultured with bovine granulosa cell monolayers and those of embryos cultured in medium alone. In this experiment, medium used for all cultures was TCM-199 supplemented with 1% FCS.

Experiment 2

The effects of serum deletion on the developmental ability of embryos cleaved to the 4-cell or 5- to 8-cell stage 24 h after culture or co-culture were examined. In this experiment, the medium used for culture and co-culture was TCM-199 supplemented either with 1% FCS or 0.1% polyvinylpyrrolidone. The embryos at each cleavage stage were transferred separately to each culture drop with or without mouse ampullae and were then cultured for 7 d. All ampullae used for co-culture were prepared at 2 to 3 h before the start of co-culture.

Statistical Analysis

In all experiments, data obtained from each replicate were combined and expressed as 0-1 variables and were analyzed by GSK (Grizzle, Starmer and Koch) method (16) using CATMOD procedure of the statistical analysis system (SAS Institute, Cary, NC). A value of $P<0.05$ was considered statistically significant.

RESULTS

Experiment 1

Development of bovine IVM/IVF embryos was successfully accomplished by co-culture with isolated mouse ampullae. As shown in Table 1, 42.9% of cleaved embryos

developed to the blastocyst stage when co-cultured with the same ampullae for 8 d. The developmental rate was higher than that obtained in granulosa cell co-culture ($P<0.05$) or in TCM-199 + 1% FCS control ($P<0.001$). When the embryos were co-cultured with mouse ampullae replaced with fresh ones at intervals of 48 h, 41.5% of the cleaved embryos developed to the blastocyst stage, although the blastulation rate was not significantly different ($P=0.06$) from that of those co-cultured with granulosa cell monolayers.

Experiment 2

Bovine IVM/IVF embryos could develop to the blastocyst stage even without serum, when co-cultured with mouse ampullae (Figure 1). As shown in Table 2, the blastulation rate (33/125; 26.4%) of oocytes co-cultured in serum deleted medium was not statistically different from that of oocytes developed in a serum-supplemented co-culture system (39/127; 30.7%). In the absence of mouse ampullae, however, the blastulation rate of embryos developed without serum was significantly lower than that of embryos developed with serum (2/120;1.7% vs 9/127;7.1%). Cleavage rates were similar in all treatments (75.0%-83.2%), and consequently there was no significant difference in the developmental rates to the 4-cell stage or to the 5- to 8-cell stage between treatments. The developmental potency of 4-cell and 5-8-cell embryos cleaved until 46 to 48 h after insemination were not significantly different irrespective of serum supplementation to the culture medium when the embryos were co-cultured with ampullae (37.0% vs 33.8% or 38.5% vs 44.6%).

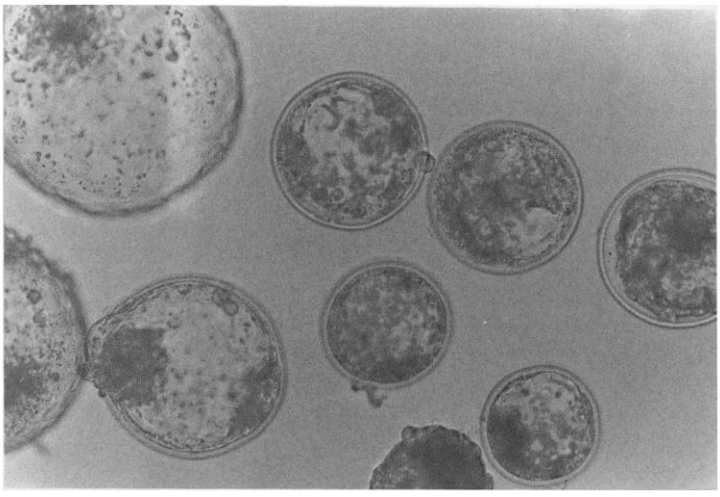

Figure 1. Blastocysts developed in co-culture with mouse ampullae under serum-free condition.

Table 1. Effects of mouse ampullae on the development of bovine IVM/IVF oocytes cultured in vitro

Treatment	Number of embryos cultured	Number of replicates	Number (%) of embryos cleaved	Number of blastocysts/ number of cleaved embryos	Mean ± SD
Mouse ampullae[a]	150	5	91(60.7)[c]	39/91(42.9)[d]	39.9±17.7
Mouse ampullae[b]	150	5	94(62.7)[c]	39/94(41.5)[d,e]	41.9±15.7
Granulosa cell	148	5	92(62.2)[c]	26/92(28.3)[e]	24.1±14.9
Control	144	5	95(66.0)[c]	4/95(4.2)[f]	3.8± 4.3

[a]Ampullae were continuously cultured for 8 days.
[b]Embryos were transferred to medium containing fresh ampullae at intervals of 48 hours.
[c-f]Different superscripts differ significantly within same column ($^{de}P<0.05$, $^{df,ef}P<0.001$).

DISCUSSION

In the present study, we demonstrated that bovine IVM/IVF oocytes could develop to the blastocyst stage when co-cultured with mouse ampullae; we also indicated that serum deletion from the culture medium had no effect on the development of embryos to blastocysts when the embryos were co-cultured with mouse ampullae. As shown in Table 1, the percentage (42.9%) of cleaved embryos developed to blastocysts in co-culture with mouse ampullae was significantly higher than that obtained in co-culture with granulosa cells (28.3%) or in culture without cells (4.2%). From our results, mouse ampullae can be successfully used for co-culture of bovine IVM/IVF oocytes to the blastocyst stage. Although the replacement of mouse ampullae at intervals of 48 h during the co-culture period was not required for the embryonic development, the beneficial effects of isolated mouse ampullae on the development of bovine IVM/IVF oocytes were exerted for at least 8 d (Table 1). The same effects were also observed under serum-free co-culture conditions. Recently, however, Sparks et al. (32) reported that bovine 1-cell embryos fertilized in vivo failed to develop beyond the 8-cell stage when cultured in explanted mouse oviducts maintained in organ culture system. They suggested that insufficient metabolism of glucose by the mouse oviducts in TCM-199 could not provide a low glucose environment for bovine embryo development. However, we used the same medium and the same oviduct in the estrous cycle. As organ culture is very sensitive culture system, environmental differences between the above mentioned

Table 2. Effects of serum deletion on the development of bovine IVM/IVF oocytes cultured with or without mouse ampullae[a]

Treatment	Number of embryos cultured	Number (%) of embryos cleaved	Number (%) of embryos cleaved to		Number (%) of blastocysts/ number of embryos cleaved to		Number (%) of blastocysts/ number of embryos cultured	Mean ± SD
			4-cell stage	5 to 8-cell stage	4-cell stage	5 to 8-cell stage		
TCM-199 + 0.1% PVP	120	90(75.0)[b]	25(20.8)[c]	-	0/25(0.0)[e]			
			-	57(47.5)[d]		2/57(3.5)[e,f]	2/120(1.7)[j]	1.1± 1.2
TCM-199 + 1% FCS	127	104(81.9)[b]	38(29.9)[c]	-	2/38(5.3)[e,g]			
			-	59(46.5)[d]		7/59(11.9)[e,g]	9/127(7.1)[j]	6.4± 4.4
TCM-199 + 0.1% PVP + ampullae	125	104(83.2)[b]	27(21.6)[c]	-	10/27(37.0)[h]			
			-	68(54.4)[d]		23/68(33.8)[h]	33/125(26.4)[k]	26.9± 5.2
TCM-199 + 1% FCS + ampullae	127	102(80.3)[b]	26(20.5)[c]	-	10/26(38.5)[h]			
			-	65(51.2)[d]		29/65(44.6)[h,i]	39/127(30.7)[k]	31.1±11.0

[a]Data were obtained from 4 replicates.

[b-k]Different superscripts differ significantly within same column ([eh]$P<0.05$, [gh]$P<0.01$, [fh,fi,gi,jk]$P<0.001$).

PVP=polyvinylpyrrolidone; FCS=fetal calf serum

organ culture system and our co-culture system may have caused the conflicting results.

Xu et al. (36) suggested that factor(s) from serum affect embryo development directly and/or through the oviduct cells, since few embryos developed beyond 8- to 16-cells in serum-free TALP with bovine oviductal epithelial cells. On the contrary, our results indicate that the co-cultured mouse ampullae can compensate for some factors required for the development through the 8- to 16-cell stage to blastocyst formation. Although the cleavage rates to the 4-cell or the 5- to 8-cell stage at 46 to 48 h after insemination were not different between serum-free culture conditions, the blastulation rates were higher in co-culture. This suggests that although the embryos may not be sufficiently influenced by the ampullae for the development to the 4- to 8-cell stage, the beneficial effects of ampullae become evident through development to the blastocyst stage. Since this effect is sustained in serum-free culture condition (Table 2), it is possible to determine whether the beneficial factor(s) from ampullae act before or beyond the 8-cell stage in the further development of embryos.

In the mouse, oviductal influences for the further development of embryos become evident at the late 2-cell stage (27), at which time control of transcription shifts from the maternal to the embryonic genome (3,5,10). Since this shift is observed at the 8- to 16-cell stage in bovine embryos (11,33), it is inferred that in bovine embryos, the oviductal influences appear at this stage when embryonic genome activation occurs. Pinyopummintr and Bavister (30) indicated that serum caused inhibition of development at or before first cleavage division but stimulated morula compaction and blastocyst formation. However, our results indicate that early cleavage of embryos can be accomplished, irrespective of the presence of serum and/or the co-existence of ampullae (Table 2). It has also been reported that a high concentration of serum (2.5 to 10%) suppresses the cleavage rate up to the 4-cell stage (19), and that decreased concentrations of FCS from 10 to 2% improve morula and blastocyst development (28). This controversy with the former may be due to the difference in concentration and/or treatment (heated or unheated) of serum used in the culture; our data were obtained from the culture conditions using a low concentration of heat-treated serum (1%), whereas Pinyopummintr et al. used 10% of unheated serum (30).

The developmental ability of embryos cleaved to the 4-cell stage at 46 to 48 h after insemination was comparable to that of embryos developed beyond the 4-cell stage at this time (Table 2). However, the embryos cleaved to the 2- to 3-cell stages until 46 to 48 h after insemination could not develop beyond the 8-cell stage (data not shown). From these results, IVM/IVF oocytes are needed to develop to at least 4-cell stage by 46 to 48 h after insemination.

In conclusion, we have demonstrated that mouse ampullae can stimulate the development of bovine IVM/IVF embryos to the blastocyst stage even when the serum was excluded from the co-culture conditions, and it is confirmed that the oviductal effects on embryonic development are not species-specific. Although serum supplementation is usually required to maintain somatic cell co-culture, the beneficial effects of mouse

ampullae in our co-culture system could be sustained for at least 8 d without serum supplementation. In addition, since maturation was also performed without serum and BSA supplementation, the mechanism of the beneficial role of oviducts in the development of bovine IVM/IVF oocytes might be elucidated without the influence of serum using our co-culture system, and eventually it may be possible to determine the relationship between the oviductal factor(s) and the occurrence of embryonic genome activation in bovine embryos.

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