Co-Culture of In Vitro Fertilized Bovine Embryos with Different Cell Monolayers

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ABSTRACT: The effects of different cell monolayers on in vitro development of early bovine embryos derived from in vitro maturation and fertilization were examined in this study. Early embryos (four to eight cells) were randomly allocated to bovine granulosa cell (GC), oviductal cell (OC), or uterine cell (UC) monolayers in Exp. 1 and to GC, skin cell (SC; from 10-d-old chicken embryos), testicular cell (TC; from 10-d-old mouse), and liver cell (LC; from 10-d-old chicken embryos) monolayers in Exp. 2, and cultured for 6 d at 38.6°C in a humidified atmosphere of 5% CO2 in air. The culture medium was 12.5 mM HEPES TCM 199 supplemented with 1% calf serum and 1 mM sodium pyruvate. In Exp. 1, the percentage of four- to eight-cell embryos that developed to blastocysts on GC, OC, and UC monolayers was 26.9 (28/104), 37.5 (39/104), and 39.2 (40/100), respectively. In Exp. 2, the percentage of four- to eight-cell embryos that developed to blastocysts on GC, SC, TC, and LC monolayers was 53.3 (40/75), 42.9 (33/77), 49.3 (37/75), and 44.3 (35/79), respectively. There were no significant differences in development among groups in either experiment. Embryos co-cultured with bovine OC developed faster, and those co-cultured with mouse TC developed slower, because the percentage (100 × number of blastocysts obtained by d 5/number of blastocysts obtained by d 6) of blastocysts developing by d 6 of blastocysts developing by d 5 of culture on GC, OC, and UC monolayers was 50.0 (14/28), 82.0 (32/38), and 67.5 (27/40), respectively, and those on GC, SC, TC, and LC monolayers were 80.0 (32/40), 81.8 (27/33), 48.6 (18/37), and 74.3 (26/35), respectively. The results of this study suggest that the embryotrophic or embryo-regulating factors may not be strictly oviduct- and species-specific.

Key Words: Bovine, Embryos, In Vitro Fertilization, Culture Techniques

Introduction

The requirements for development of early embryos of domestic animals have not been defined (Rexroad, 1989). Somatic cell co-culture is a means of overcoming the in vitro developmental block of cultured mammalian embryos. Tissues from the female reproductive tract and other sources have been used to support the development of embryos. These include in vitro co-culture with feeder cells (Cole and Paul, 1965), fibroblast monolayers (Kuzan and Wright, 1982; Voelkel et al., 1985), trophoblastic vesicles (Camous et al., 1984; Heyman et al., 1987; Pool et al., 1988), oviductal cell monolayers (Eyestone et al., 1987), and cumulus cell monolayers (Kajihara et al., 1987; Goto et al., 1988). Recent successes in producing calves from in vitro matured, fertilized, and cultured oocytes (Goto et al., 1988; Lu et al., 1988; Eyestone and First, 1989) have increased the importance of early embryo culture.

The objective of this study was to evaluate the effects of different cell monolayers on in vitro development of early bovine embryos produced by in vitro maturation and fertilization.

Materials and Methods

Collection of Oocytes. Ovaries were collected from nonpregnant cows of various stages of the estrous cycle at a local slaughterhouse and brought to the laboratory in physiological saline (85% w/w NaCl) at 32 to 38°C within 4 h. Follicular oocytes (5 to 25 per ovary) were collected by puncturing follicles with diameters of 1 to 7 mm with a needle (Goto et al., 1988).
Culture of Oocytes. Precise details of our procedures for the culture of cumulus-oocyte complexes (COC) have been described previously (Goto et al., 1988). After washing the oocytes twice with Whittingham’s modified Dulbecco’s phosphate-buffered saline (mPBS; Whittingham, 1971) supplemented with 2% calf serum (Gibco, Grand Island, NY) and once with the maturation medium (12.5 mM HEPES TCM 199 with Earle’s salt [Gibco] supplemented with 5% calf serum), the oocytes (approximately 70 oocytes per dish) covered by cumulus cells on more than one-third of their surface were placed into maturation medium (2.5 mL) covered with mineral oil (Squibbs & Sons, Princeton, NJ) in polystyrene culture dishes (35 mm, Termo, Tokyo, Japan) and cultured for 19 to 22 h in a CO₂ incubator (5% CO₂ in air) at 38.6°C. The same maturation protocol was used throughout this study.

Sperm Preparation. Frozen sperm of epididymal origin were prepared according to our previous report (Goto et al., 1988). Plastic straws of epididymal sperm were thawed in warm water (35°C) and then washed three times by centrifugation (600 x g for 5 to 7 min) with BO medium (10 mL; Brackett and Oliphant, 1975) without BSA but supplemented with 5 mM caffeine. Spermatozoa were preincubated for 2 to 3 h at 38.6°C (5% CO₂ in air) in a small test tube in BO medium (1 to 2 mL) containing BSA (5 mg/mL; A-4387, Sigma Chemical, St. Louis, MO) and caffeine (2.5 mM). After preincubation the tube was gently shaken and small (110-μL) sperm microdrops (2 x 10⁶ cells/mL) were prepared for insemination. The microdrops were covered with mineral oil.

Insemination and Subsequent Culture. The method used was similar to our previous report (Goto et al., 1988). After incubation for 19 to 22 h the cumulus-oocyte complexes were transferred to the sperm microdrop (15 to 20 oocytes per microdrop). After 6 h of incubation with sperm, cumulus-oocyte complexes were washed with development medium to remove excess sperm attached to the complexes, and complexes were transferred into development medium (12.5 mM HEPES TCM 199 supplemented with 1% calf serum and 1 mM sodium pyruvate) and cultured for 66 h for further development. The culture medium (2.5 mL) in a polystyrene dish (35 mm) was covered with mineral oil.

Preparation of Different Cell Monolayers. Granulosa cell monolayers were prepared from granulosa cells aspirated from bovine follicles (1 to 7 mm) according to our previous report (Goto et al., 1990a). Follicular fluid containing granulosa cells was centrifuged at 180 x g for 5 min. The pellet of granulosa cells was washed by centrifugation for 5 min in 5 mL of mPBS supplemented with 1% calf serum. The pellet was suspended in an enzyme solution (1 mL) containing protease (Dispase, 1,000 U/mL of PBS, Sanko Junyaku, Tokyo, Japan). Cells were incubated for 20 to 40 min at 38.6°C. The cells were then vigorously pipetted to permit the separation of cells followed by washing (180 x g for 5 min, three times) in mPBS (5 mL). After the final washing, 5 mL of culture medium (12.5 mM HEPES TCM 199 supplemented with 5% calf serum) was added to the cells and the cell suspension was passed through wire mesh to remove cell debris. Cell concentration was adjusted to 10⁶ cells/mL by adding culture medium. One and one-half milliliter volumes of cell suspension were then placed in 12-well plates (Coster, Cambridge, UK) so that they would reach 80 to 100% confluence within 2 d. Oviducts and uteri were obtained from cows shortly after slaughter at a local slaughterhouse and transported to the laboratory (32 to 38°C) within 2 h. Although we did not examine the estrous cycle of cows before they were killed, oviducts and uteri were obtained from cows with newly formed corpora lutea at the time of slaughter in each trial. Oviducts were straightened by dissection and the cells were collected by stripping epithelial cells from the ampulla by using a glass slide. Uteri were dissected and cells were collected by stripping epithelial cells from the uterus close to the oviduct. The extruded cells were washed with mPBS (5 mL) by centrifugation (180 x g for 5 min). The pellet of cells was suspended in an enzyme solution described above. The following procedures were the same as those described for granulosa cells, and the prepared cell suspensions (10⁵ cells/mL) were placed in 12-well plates so that they would reach 80 to 100% confluence within 5 to 7 d.

Skin and liver were recovered from a 10-d-old chicken embryo and testis was secured from a 10-d-old mouse. Each tissue was cut into small pieces (approximately 1 mm²) and treated with the enzyme solution mentioned above. The following procedures were the same as those described for granulosa cells, and the prepared cell suspensions (10⁵ cells/mL) were placed in 12-well plates so that they would reach 80 to 100% confluence within 4 d.

A sample of each type of cells was stained with trypan blue (0.4% solution) to assess viability. Percentages of live cells were > 80% at the start of culture.

Co-Culture of Embryos with Different Cell Monolayers. Seventy-two hours after insemination (66 h after culturing in the development medium), cumulus cells surrounding embryos were removed by pipetting and the developmental stage of embryos was examined. Embryos that developed into four to eight cells were randomly allocated to granulosa cell (GC), oviductal cell (OC), and uterine cell (UC) monolayers in Exp. 1 and to GC, skin
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Table 1. In vitro development of bovine four- to eight-cell embryos to blastocyst stage on different cell monolayers, Exp. 1a

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Days of culture</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Granulosa cell</td>
<td>5/28</td>
</tr>
<tr>
<td>(%)</td>
<td>(17.9)</td>
</tr>
<tr>
<td>Oviductal cell</td>
<td>8/39</td>
</tr>
<tr>
<td>(%)</td>
<td>(20.5)</td>
</tr>
<tr>
<td>Uterine cell</td>
<td>11/40</td>
</tr>
<tr>
<td>(%)</td>
<td>(27.5)</td>
</tr>
</tbody>
</table>

aSum of three trials.
b,cValues not having a common superscript letter in the same column differ (P < .05).

cell (SC), testicular cell (TC), and liver cell (LC) monolayers and medium alone (only one trial) in Exp. 2 and cultured at 38.6°C in a CO2 incubator. Embryos were transferred to co-culture when each type of cells had reached 80 to 100% confluence. The culture medium used for co-culture was 12.5 mM HEPES TCM 199 supplemented with 1% calf serum and 1 mM sodium pyruvate. The culture period for each experiment was 6 d. The incubation medium was replaced with new medium every 48 h. All the media used were supplemented with antibiotics (100 IU of penicillin/mL + 100 µg/mL of streptomycin-1·mL-1). Each experiment was repeated three times except one group (medium alone) in Exp. 2. Experiments 1 and 2 were conducted in 1989 and 1990, respectively.

Statistical Analysis. The chi-square test was used to test for differences in development among groups.

Results

Experiment 1. Percentage of four- to eight-cell embryos that developed to morulae on GC, OC, and UC monolayers was 49.0 (51/104), 45.2 (47/104), and 50.0 (51/102), respectively. Percentage of the four- to eight-cell embryos that developed to blastocysts on GC, OC, and UC monolayers was 26.9 (28/104), 37.5 (39/104), and 39.2 (40/102), respectively. There were no significant differences in development among groups. However, embryos co-cultured with GC or UC developed faster than those co-cultured with SC. Therefore, we did not check them histochemically, oviductal and uterine cells were predominantly epithelial cells under microscopic examination.

Experiment 2. Percentage of four- to eight-cell embryos that developed to morulae on GC, SC, TC, and LC monolayers was 58.7 (44/75), 54.5 (42/77), 57.3 (43/75), and 55.7 (44/79), respectively. Percentage of four- to eight-cell embryos that developed to blastocysts on GC, SC, TC, and LC monolayers was 53.3 (40/75), 42.9 (39/77), 49.3 (37/75), and 44.3 (35/78), respectively. There were no significant differences in development among groups. However, embryos co-cultured with TC developed more slowly than those of GC, SC, and LC, because 48.6% (18/37) of embryos became blastocysts within 5 d of culture in the TC group, compared with 80.0% (32/40), 81.8% (27/33), and 74.3% (26/35) within the same period in the GC, SC, and LC groups, respectively (Table 2). In contrast, only 23.1% (6/28) of embryos developed to blastocysts in medium alone (without co-culture) and their morphological quality was poor (unclear inner cell mass; more dead or extruded cells) compared with blastocysts obtained in co-culture (data are not shown in Table 2 due to only one trial). Although we did not check them histochemically, testicular and liver cells were predominantly parenchymal cells under microscopic examination.

Discussion

Results of this study demonstrate that four- to eight-cell bovine embryos can develop to blastocysts at a similar rate on different types of cell monolayers in vitro under the present culture condition. Embryos co-cultured with bovine oviductal cells developed faster, whereas those co-cultured with mouse testicular cells developed more slowly. The positive effect exerted by the reproductive tract cells may be nonspecific because other types of cells (skin, testicular, liver) from a different embryologic origin of different

Table 2. In vitro development of bovine four- to eight-cell embryos to blastocyst stage on different cell monolayers, Exp. 2a

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Days of culture</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Granulosa cell</td>
<td>11/40</td>
</tr>
<tr>
<td>(%)</td>
<td>(27.5)</td>
</tr>
<tr>
<td>Skin cell</td>
<td>16/33</td>
</tr>
<tr>
<td>(%)</td>
<td>(48.3)</td>
</tr>
<tr>
<td>Testicular cell</td>
<td>11/37</td>
</tr>
<tr>
<td>(%)</td>
<td>(39.7)</td>
</tr>
<tr>
<td>Liver cell</td>
<td>11/35</td>
</tr>
<tr>
<td>(%)</td>
<td>(31.4)</td>
</tr>
</tbody>
</table>

aSum of three trials.
b,c,dValues not having a common superscript letter in the same column differ (P < .05).
species supported development of bovine embryos in vitro in this study. This result is not surprising because other scientists have also reported positive effects of co-culture systems on development of embryos using various types of cells. Kuzan and Wright (1982) found that co-culture of bovine embryos with bovine fibroblasts promoted embryo hatching and attachment in vitro. Camous et al. (1984) showed that successful culture through the 8- to 16-cell block required the presence of an active biological component such as trophoblastic vesicles. Voelkel et al. (1985) found that a bovine endometrial fibroblast monolayer system was excellent for culture of halved bovine embryos. Eyestone et al. (1987) co-cultured early bovine embryos (five to eight cells) on oviduct cell monolayers and found that co-cultured embryos developed to the late morula or blastocyst stage, whereas embryos cultured in medium alone failed to develop beyond 16 cells. In our study, 23.1% (6/26) of four- to eight-cell embryos developed to blastocysts in medium alone; however, their morphological quality was poor compared with embryos produced by co-culture. These results suggest that co-culture systems not only support embryonic development but also improve the quality of embryos produced in vitro.

Jiang et al. (1991) evaluated effects of different cell monolayers on in vitro development of early bovine embryos (two- to eight-cell). They compared the in vitro development of embryos on seven different monolayers of bovine origin (GC, OC, UC, and combinations of these cells) and found no differences in development to blastocysts except one (between granulosa and uterine cells, 37.2 vs 26.1%). In the present study, we used different cell monolayers from different embryologic origin and from different species and found no differences in development to blastocysts.

There are papers suggesting that the beneficial effects of oviductal cells are relatively specific. Sheep embryonic fibroblasts (Gandolfi and Moor, 1987), uterine cells and kidney cells (Rexroad and Powell, 1988a), and trophoblastic vesicles (Rexroad and Powell, 1988b) failed to stimulate in vitro cleavage of early-stage sheep embryos to the same degree as oviductal cells. Aoyagi et al. (1990) reported that co-culture of bovine embryos (eight-cell) with trophoblastic vesicles, amniotic sac cells, oviductal cells, or cumulus cells supported development of bovine embryos in vitro and they observed differences in development among cell types.

Because our study was conducted over 2 yr (Exp. 1 in 1989; Exp. 2 in 1990), we used granulosa cell monolayers as a control. This was the system by which we had first obtained calves (Goto et al., 1988). Although we do not have good explanations, apparent differences between Exp. 1 and Exp. 2 in the percentage (26.9 vs 53.3%) of embryos that developed to blastocysts on granulosa cell monolayer may come from improvement of our technique over 2 yr or may come from variations in age, nutritional status, or other factors of cows that provided ovaries at the local slaughterhouse. We had observed that oocytes from individual cows differed in their ability to develop to blastocysts in vitro (Goto et al., 1990b).

In conclusion, we found no differences in in vitro development of early bovine embryos on different cell monolayers from different species, but embryos co-cultured with bovine oviducal cells developed faster. The results of this study, in conjunction with others, suggest that the embryotrophic or embryo-regulating factors may not be strictly oviduct- and species-specific under the present culture condition. However, further improvements in the culture system to make it more similar to the in vivo system may find differences in embryonic development among cell types.

Implications

The results of this study provided an additional observation concerning a co-culture system for the development of early bovine embryos in vitro. The results support the idea that the embryotrophic or embryo-regulating factors from co-cultured cells may not be strictly oviduct- and species-specific.

Literature Cited


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