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Bovine 1–2-Cell Embryo Development Using a Simple Medium in Three Oviduct Epithelial Cell Coculture Systems[1]

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ABSTRACT

These studies were designed to develop a coculture system using a simple medium to promote development of 1-cell bovine embryos through the 8–16-cell stage to morula and blastocyst stages. Monolayers for coculture were prepared from bovine oviduct epithelial cells (BOEC). In vivo-fertilized 1–2-cell embryos and ova (384) were surgically collected from superovulated cows. In Experiment 1, embryos cocultured in a simple glucose-free and serum-free medium (CZB) developed with superior scores of embryo quality than embryos cocultured in Ham's F-10 with serum, and a greater percentage developed past 8–16 cells than embryos cocultured in CMRL-1066 with serum ($p < 0.05$). In Experiment 2, embryos cocultured with fresh BOEC monolayers averaged more ($p < 0.05$) cells than did embryos in coculture with frozen-thawed BOEC monolayers or in BOEC-conditioned medium. Without glucose in the simple medium for the first 48 h of culture, more embryos blastulated ($p < 0.01$) by Day 5.5 of culture (Day 6.5 of donor's estrous cycle) than embryos in the same medium with glucose present throughout. In Experiment 3, more embryos tended to hatch in BOEC coculture ($p < 0.10$) than in conditioned medium. These results show that a chemically simple medium with fresh BOEC monolayers can provide a significant benefit for coculture of early bovine embryos.

INTRODUCTION

In many species a major obstacle to the in vitro study of early embryonic development and the application of biotechnologies, such as in vitro fertilization, microinjection of individual sperm, and gene transfer, has been the cleavage "block" that occurs in vitro with conventional culture methods. In cattle, this event occurs at the 8–16-cell stage (Wright and Bondoli, 1981; Camous et al., 1984; Eyestone and First, 1986). In order to develop bovine zygotes past 8–16 cells to stages where nonsurgical embryo transfer to recipients can occur, researchers have begun to investigate the role of embryo-somatic cell coculture (Heyman et al., 1987; Eyestone and First, 1989; Rexroad, 1989).

The use of embryo coculture with trophoblast, granulosa, fibroblast, luteal, endometrial, oviductal, or other cell types has invariably afforded some benefit over the use of medium alone for bovine embryo development (Voekle et al., 1985; Heyman et al., 1987; Bavister, 1988; Eyestone and First, 1989; Goto et al., 1989). In developing a coculture system for studying physiological processes during embryo development, it is appropriate to choose cells originating from organs where these events occur in vivo. Oviduct epithelial cells have supported embryonic development past the in vitro culture block in several species, including sheep and cattle, and have afforded some benefit over other cell types (Gandolfi and Moor, 1987; Rexroad and Powell, 1988). Investigators (Rexroad and Powell, 1988; Eyestone and First, 1989) have also begun to evaluate the use of oviduct-cell-conditioned medium or frozen-thawed oviduct cells. Such approaches provide a way to study the mechanisms of oviduct cell-embryo interactions, and have the advantage over fresh cultures of greater convenience and uniformity.

Recent work in mice and hamsters has suggested that the presence of glucose in early embryonic stages is deleterious to future embryo development (Chatot et al., 1989; Sheshagiri and Bavister, 1989). A simple glucose-free, serum-free medium containing pyruvate, lactate, glutamine, and bovine serum albumin (BSA) was developed by Chatot et al. (1989) to overcome the in vitro culture 2-cell block in mice; it has been named CZB (Chatot, Ziomek, Bavister) medium by the authors. In cattle, biochemical studies of the blastocyst have suggested that glycolysis is blocked at this stage because of a possible lack of pyruvate kinase, but that glutamine can be utilized as an energy source (Rieger and Guay, 1988). Similar studies on earlier-stage bovine embryos are lacking.

In much of the previous culture work on bovine embryos, Ham's F-10 (GIBCO, Grand Island, NY) with fetal bovine serum (FBS) has been used as the nutrient medium (Wright and Bondoli, 1981; Rexroad, 1989). Recently several researchers (Bates et al., 1985; Eyestone and First, 1989; Goto et al., 1989) have begun using more complex media, such as TCM199 or CMRL-1066 (GIBCO, Grand Island, NY), with higher amino acid, vitamin, and cofactor levels, also supplemented with FBS or other sera in varying amounts. The use of different batches and types of serum in most culture media is a source of variability and also complicates

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analysis of conditioned media for oviduct-cell-derived proteins or other molecules.

Consequently, experiments were designed to evaluate early bovine embryo development in coculture with the simple serum-free and glucose-free CZB medium. The objectives were to compare (1) embryonic development in CZB medium versus complex serum-containing media, (2) the effect of CZB medium with and without glucose on early bovine embryo development, and (3) the ability of medium conditioned with oviduct epithelial cells, of frozen-thawed cell monolayers, and of fresh cell monolayers to support embryonic development in CZB.

MATERIALS AND METHODS

Establishment of Oviduct Cell Monolayers

Bovine oviduct epithelial cells (BOEC) were surgically obtained by cannulation and lavage of oviducts from superovulated embryo donor cows (as described below), using phosphate-buffered saline (osmolality = 280 mOsmol/kg H_2O) containing 0.1% polyvinyl alcohol and 100 U/ml penicillin G, 0.10 mg/ml streptomycin, and 0.25 μg/ml amphotericin B (GIBCO). Oviduct cells were recovered 40–48 h after the beginning of standing estrus. Recovered oviduct cells were present both as single cells and as rafts of large cell clumps showing movement due to ciliary activity, as described by others (Joshi, 1988; Eyestone and First, 1989). A 0.25-ml pellet of these cells, obtained by gravity sedimentation, was resuspended in 5 ml of Ham's F-10 supplemented with 10% FBS plus antibiotics and antimycotic as above, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium (ITS), and 10 ng/ml epidermal growth factor (EGF; Collaborative Research, Lexington, MA). This medium is referred to as HFFBS+. The cell suspension was then equally dispensed as 0.5-ml volumes into 4-well tissue culture plates (Nunclon, Thomas Scientific, Swedesboro, NJ) and cultured at 39°C in 5% CO_2 in humidified air. Twenty-four to 36 h after the start of culture, medium and unattached cells were removed, and 0.5 ml of fresh HFFBS+ was applied. Confluent monolayers with some ciliary activity formed within 4–6 days and were used for coculture within 14 days.

Freezing of Oviduct Cells

Recently formed BOEC monolayers (1–2 wk of age) that had previously supported bovine zygote development were covered with a solution containing 2.5 mg/ml trypsin and 0.2 mg/ml EDTA. After approximately 15 min of incubation, the oviduct cells were washed off the culture plates with HFFBS+, and sterile FBS was added to make a solution of 50% serum and 50% Ham's F-10. Medical-grade dimethylsulfoxide, (DMSO, Syntex, West Des Moines, IA) was then slowly added over a period of 2 min to reach a final concentration of 10% of the total volume (Munson, 1988). This cell suspension was then rapidly dispensed into freezing vials and placed in a sealed styrofoam container which was stored at −70°C overnight. The next morning, freezing vials were plunged directly into liquid nitrogen and stored in liquid nitrogen tanks for at least 30 days. Vials were thawed in a 37°C water bath. The thawed BOEC were washed two times in the supplemented Ham's F-10 by centrifugation to remove DMSO.

Collection of 1–2 Cell Bovine Embryos

Holstein virgin and first-calf heifers were superovulated using a total of 20 mg of follicle-stimulating hormone (FSH; Folltropin, Vetrepharm, London, ON, equivalent to 35 mg NIH-FSH-S-1 standard) beginning on Days 8–12 of their cycle. The FSH was given every 12 h over 4 days, and 750 μg of cloprostenol (Estrumate, Bayvet, Shawnee, KS) was given along with the seventh FSH injection. Animals were evaluated for estrus at 0800 h and 2000 h each day. Cattle displayed estrus 36–48 h after cloprostenol injection and were bred every 12 h after the start of standing estrus until 24 h after the end of estrus (from 2–4 inseminations). Surgical collection of embryos and oviduct cells was done 40–48 h after the start of standing estrus. Animals were anesthetized with thiamylal sodium and maintained on halothane inhalant. A midline ventral incision was performed to exteriorize and lavage both oviducts as previously described (Ellington et al., 1990a). The embryos and ova were transported in collection medium to the laboratory, where they were isolated, examined for developmental stage, washed three times in fresh phosphate-buffered saline and then placed into culture within 1 h of collection. No effort was made to differentiate unfertilized oocytes from zygotes at this time.

Embryo Culture and Evaluation

All embryos were cultured at 39°C in 5% CO_2/95% humidified air. Embryos were evaluated daily, and embryo quality scores were assigned using the commercial embryo transfer scale: excellent = 1, good = 2, fair = 3, poor = 4, and degenerate = 5 (Hasler et al., 1987). At the end of the culture period, stage of embryonic development was assessed, and embryos were fixed in 1% formalin for subsequent determination of cell numbers. To count cell nuclei, embryos were stained with Hoechst 33342 DNA stain and viewed under an epifluorescence microscope (Pursel et al., 1985). Nuclei in each embryo were counted and assigned to one of 3 categories based on morphologic appearance: healthy-appearing interphase nuclei; nuclei with condensed chromatin that appeared to be in mitosis; and nuclei appearing as a small or fragmenting, densely stained pyknotic mass (Fig. 1). Nuclei in the third category were considered to be of poor quality, although it is possible that a small percentage of healthy nuclei in the final stages of telophase may have occasionally been placed in this category because of their similar appearance.

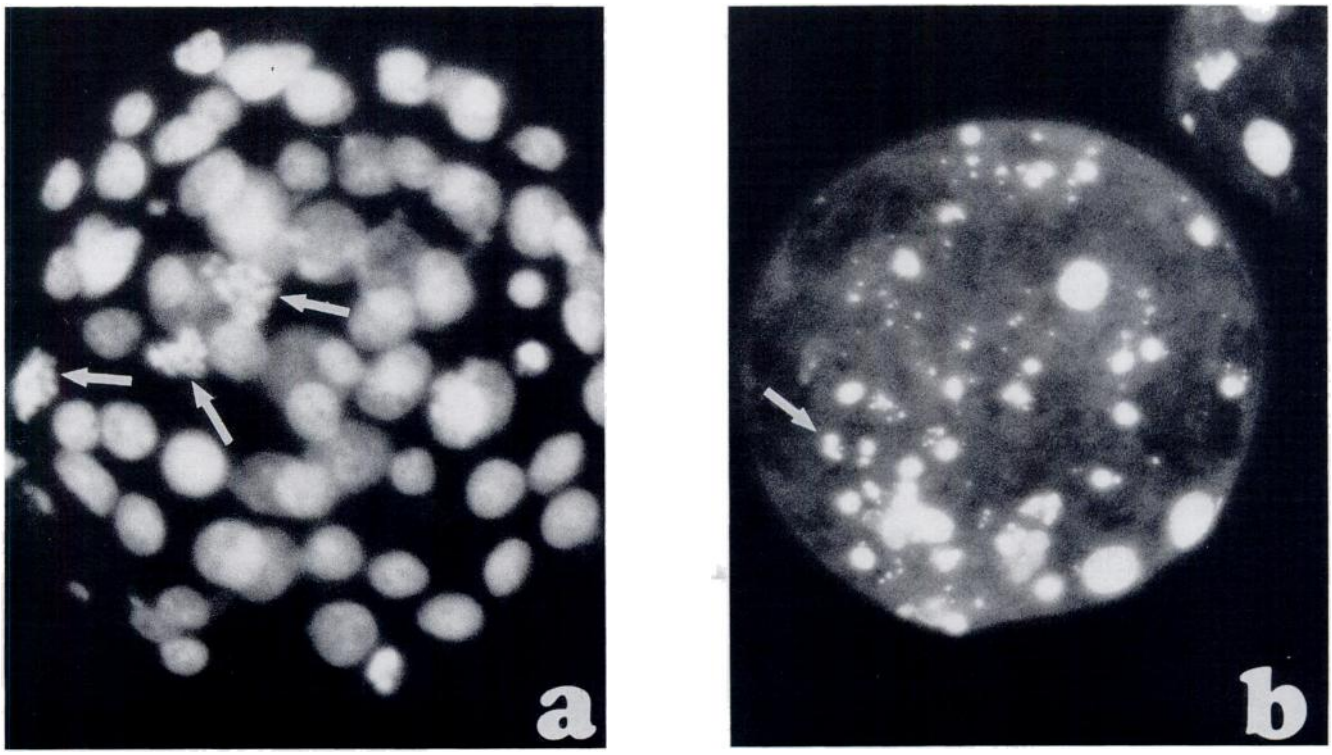

FIG. 1. (a) Healthy early blastocyst after 5.5 days of coculture in CZB medium. Note cells showing mitotic activity (arrows). × 80. (b) Very poor morula after 5.5 days of coculture in CZB medium. Many nuclei are fragmented and/or are pyknotic (arrow). × 50.

Experiment 1a

This experiment used a 2 × 3 factorial design to compare embryonic development with and without BOEC coculture, using two complex serum-containing media and a simple serum-free medium. The CMRL-1066, a complex culture medium containing 58 components, was compared to Ham's F-10 containing 46 components, and CZB, a glucose- and serum-free medium of 13 components including pyruvate, lactate, glutamine, and BSA (Chatot et al., 1989).

Eighty-seven embryos and ova surgically recovered from 7 donor cows were distributed across the six treatments. For efficient use of the embryos, the number of embryos in Ham's F-10 and CMRL medium alone were kept to a minimum (8 in each medium), since previous results in our laboratory (Ellington, unpublished data, 1988) and from Wright and Bondoli (1981) clearly showed the inability of Ham's F-10, as well as other complex serum-containing media, to solely support bovine embryo development past the 8–16-cell stage. The CZB medium without coculture was evaluated more extensively (25 embryos) because of its encouraging results in overcoming the 2-cell culture block in mice (Chatot et al., 1989).

One-cell (and, when present, 2-cell) bovine embryos from each donor cow were randomly allocated to the following treatment groups: (1) Ham's F-10 + 10% FBS (HFFBS) over a BOEC monolayer; (2) HFFBS alone; (3) CMRL-1066 + 10% FBS (CMFBS) with BOEC; (4) CMFBS alone; (5) CZB with BOEC; or (6) CZB alone. Embryos were cultured for 5 days in 0.5 ml of medium that was changed every 2 days. On Day 5, embryo stage was recorded and cells were counted, as described above.

Experiment 1b

Because of poor viability of oviduct cells in the serum-free and glucose-free CZB medium, an experiment was designed to study development of 1–2-cell bovine embryos in coculture using CZB supplemented by ITS/EGF, with glucose added after 36–48 h of culture to enhance oviduct cell viability after most embryos had developed to ≥8 cells. This medium was compared with CMFBS, also with ITS/EGF added, after embryos had developed to ≥8 cells. The ITS/EGF was added after 36–48 h of coculture because of previous results showing decreased embryo development in coculture with ITS/EGF added to both media at the beginning of culture (Ellington, unpublished data, 1988). Eighty-nine recovered embryos and ova were distributed equally from each of 8 donors into coculture in either CMFBS or CZB for 36–48 h. At this time, ITS/EGF was added to both media so that embryos were cultured the remaining 3.5 days in either (1) CMFBS + ITS/EGF (as described for monolayer preparation) or (2) CZB + ITS/EGF + 3.3 mM glucose. At the end of 5 days of culture, embryos were evaluated and stained with Hoechst 33342 DNA stain.

Experiment 2

A 2 × 3 factorial experiment was designed to compare embryonic development in CZB with and without glucose during the first 36–48 h in three coculture systems: fresh oviduct cell monolayers, frozen-thawed oviduct cell monolayers, and medium conditioned with oviduct cell monolayers. One hundred-fifty embryos and ova from 8 donors were randomly distributed by donor across the six treatments. In all treatments, culture media were changed to CZB + 3.3 mM glucose + ITS/EGF after 36–48 h when embryos were ≥8 cells. Monolayers from fresh and frozen-thawed BOEC were prepared as previously described. Conditioned medium was obtained by incubating CZB medium (with or without glucose), for 24 h with 1–2-wk-old monolayers prepared from fresh oviduct epithelial cells. Just before embryo recovery, conditioned medium was removed, centrifuged, and evaluated under an inverted phase-contrast microscope for cell presence. Any cells seen were removed by aspiration with a sterile pipette. Embryos in all treatments were cultured for 5.5 days and then stained with Hoechst 33342 and evaluated as in Experiment 1.

Experiment 3

The objective of this experiment was to evaluate the ability of zygotes cultured past the 16-cell stage in CZB using either BOEC coculture or conditioned medium to hatch after an extended time in culture. Fifty-eight 1–2-cell embryos and ova from 5 donors were randomly allocated by donor to either fresh monolayer coculture in CZB or to BOEC-conditioned medium and were cultured for 6 days. Media in both treatment groups were changed to glucose and ITS/EGF containing CZB at 36–48 h of culture. On Day 6, the medium was changed to CMFBS + ITS/EGF (as in Experiment 1), either with the coculture or as conditioned medium. This change was done to maintain monolayer viability over an extended time. Embryos were cultured another 4 days until Day 11 of the donor's estrous cycle. Embryos were evaluated daily for stage of development, especially for blastocoele expansion and/or hatching. After Day 10 of culture, embryos that had not hatched were stained to determine cell number.

TABLE 1. Development of 1–2-cell embryos in coculture for 5 days (Experiment 1a).

	Medium		
Parameter	CMRL + FBS	Ham's + FBS	CZB
No. of embryos	15	14	13
Mean cell count (Range)	25 (7–42)	29 (4–63)	29 (5–65)
Mean embryo score	2.7[a]	3.1[b]	2.7[a]
Morulae or blastocysts, %	73	79	85
Mean no. pyknotic nuclei ± SE	5 ± 1[ab]	7 ± 2[a]	4 ± 1[b]

[ab]Row means with different superscripts differ at $p < 0.05$.

Statistical Analysis

The proportions of embryos forming blastocysts or hatching blastocysts were analyzed by Chi-square analysis. Experiments comparing cell number, quality score, and number of pyknotic nuclei were analyzed by Student's *t*-test if only two treatments were compared. Experiments involving multiple comparisons were studied by analysis of variance followed by Duncan's multiple range test of the means. Means were considered significantly different at $p < 0.05$.

RESULTS

Recovery of 1–2-Cell Embryos

The embryos and ova recovered were composed of 83% zygotes and 10% 2-cell embryos. Seven percent were classified as unfertilized ova on the basis of initial appearance and failure to cleave more than once in culture. Three percent of the recovered zygotes failed to develop beyond 3 cells. These zygotes may have been abnormally fertilized and were thus not included in the experimental analysis of the culture systems. This percentage was consistent throughout all treatments.

Experiment 1a

In media without coculture, 1/8 embryos in HFFBS, 0/8 embryos in CMFBS, and 0/25 embryos in CZB developed past the 16-cell stage, with average cell counts of 8, 6, and 10, respectively. Cocultured embryos (Table 1) developed past the 16-cell stage similarly in all three treatments (≥73%), and the mean numbers of cells per embryo were also similar. However, embryos in CZB medium had significantly ($p < 0.05$) fewer pyknotic nuclei and received superior quality scores ($p < 0.05$) compared with embryos cocultured in HFFBS. The CMFBS-cocultured embryos also had a higher ($p < 0.05$) mean score for embryo quality than did embryos cocultured in HFFBS. The percentage of cells in mitosis at the time of fixation was less than 1% for all treatments and did not differ significantly ($p > 0.05$) among treatments.

Viability of the oviduct cells was severely limited in CZB medium without glucose or serum. By 72 h of culture, approximately 75% of the monolayer had sloughed off the surface of the wells and appeared to be dead. However, by that time embryos had developed past 16 cells and continued to develop, even in the presence of the dying oviduct cells. Monolayers in both HFFBS and CMFBS appeared to be in excellent condition, with many cells maintaining ciliary activity for up to 2 wk.

Experiment 1b

The CZB medium (with glucose and ITS/EGF added after embryos reached 8 cells) supported a significantly ($p < 0.05$) higher proportion of embryos through the

TABLE 2. Development of 1–2-cell embryos in coculture after 5 days (Experiment 1b).

Parameter	Medium: CMRL + FBS (with ITS/after 48 h)	Medium: CZB (with ITS/EGF and 3.3 mM glucose after 48 h)
No. of embryos	44	40
Mean cell count	31[a]	58[b]
(Range)	(5–121)	(5–187)
Mean embryo score	2.8	2.8
Morulae or blastocysts, %	68[a]	88[b]
Mean no. pyknotic nuclei ± SE	4 ± 0.5	4 ± 0.8

[ab]Row means with different superscripts differ at $p < 0.05$.

8–16-cell block (Table 2), with a higher mean cell number ($p < 0.05$) than the CMFBS (with ITS/EGF also added 36–48 h after culture began). Scores of mean embryo quality and numbers of pyknotic nuclei were identical for the two treatments. Percentage of cells in mitosis also did not differ ($p > 0.05$) between the treatments (CMFBS, 1.1%; CZB, 1.2%).

Viability of the oviduct cells appeared to be much better in CZB with glucose and ITS/EGF added after 36–48 h of culture than without. However, even with these added nutrients, approximately 20% of the monolayer had sloughed off the surface of the well by Day 5 of culture.

Experiment 2

Conditioned medium, frozen-thawed oviduct cell monolayers, and fresh oviduct cell monolayers all allowed a high proportion of embryos to develop past the 8–16-cell stage (≥86%). However, the cell numbers for embryos in conditioned medium and frozen-thawed oviduct cells were only half ($p < 0.05$) of those for embryos cocultured with fresh oviduct cells (Table 3). The absence or presence of glucose during the first 36–48 h affected neither total cell numbers nor the percentage of embryos developing past 16 cells ($p > 0.05$).

Average number of cells with poor-quality nuclei (Table 4) was similar for all treatments. However, when expressed as a percentage of cells in the embryos, those embryos in fresh BOEC coculture tended to have a lower percentage of poor-quality nuclei. This difference was significant ($p < 0.05$) between embryos cocultured in CZB with glucose in fresh BOEC and those in frozen-thawed BOEC. The proportion of poor-quality nuclei was not affected by the presence (21%) or absence (19%) of glucose.

The average embryo quality score in fresh coculture was 2.6, superior ($p < 0.05$) to that of 3.1 in conditioned medium and 3.3 in frozen-thawed coculture. Quality scores were not affected by glucose treatment; embryos cultured without glucose during the initial 36–48 h had a mean score of 2.9. Embryos cocultured with glucose throughout had a mean score of 3.0.

The mean percentage of embryonic cells in mitosis for the 6 treatments ranged from 0.5% for embryos in coculture with frozen-thawed cell monolayers in CZB without initial glucose to 1.3% for embryos in conditioned CZB medium with glucose. These differences were not significant ($p > 0.05$).

Blastocyst formation by the end of Day 5.5 (Table 5) in culture did not differ significantly between the embryo coculture systems. However, there was a significant ($p < 0.01$) inhibitory effect of glucose presence the first 36–48 h on blastulation. Embryos cultured in CZB with glucose the first

TABLE 3. Cell numbers in BOEC and embryo coculture (Experiment 2).

Coculture system	Medium: CZB + glucose throughout — n	Mean cell no. ± SE	Range cell no.	>16 cells %	Medium: CZB without glucose first 36 h — n	Mean cell no. ± SE	Range cell no.	>16 cells %
Conditioned medium	22	34 ± 3[a]	(10–77)	86	21	30 ± 4[a]	(10–94)	86
Frozen-thawed coculture	22	37 ± 5[a]	(4–86)	86	23	44 ± 4[a]	(6–125)	91
Fresh coculture	24	76 ± 9[b]	(7–189)	96	23	76 ± 11[b]	(5–169)	87
Overall mean		50 ± 4				50 ± 5		

[ab]Means in the same column with different superscripts differ at $p < 0.05$.

TABLE 4. Presence of poor quality nuclei in BOEC and embryo coculture (Experiment 2).

Coculture system	Medium: CZB + glucose — Mean no. poor nuclei/ embryo ± SE	CZB + glucose — % poor quality nuclei	CZB without glucose — Mean no. poor nuclei/ embryo ± SE	CZB without glucose — % poor quality nuclei	Overall % poor quality nuclei
Conditioned medium	7 ± 1	23[ab]	7 ± 2	23	23
Frozen-thawed coculture	6 ± 1	28[b]	6 ± 1	18	23
Fresh coculture	5 ± 1	12[a]	5 ± 1	14	13
Overall mean		21		19	

[ab]Means in the same column with different superscripts differ at $p < 0.05$.

36–48 h of culture formed fewer blastocysts (13%) than did embryos cultured without glucose during this time (31%).

Experiment 3

After 10 days in culture, more embryos had hatched in coculture with monolayers (9/27) than in conditioned medium (3/26), although the difference was not significant ($p < 0.10$). Expanded blastocysts that had not hatched by the end of Day 9 of culture (Day 10.5 of donor's estrous cycle) never hatched (11/27 blastocysts in coculture and 5/26 in conditioned medium). The remaining 7 embryos in coculture and 18 embryos in conditioned medium failed to develop to the expanded-blastocyst stage. Comparable percentages of embryos developed beyond the 16-cell stage in both groups (93% fresh coculture, 92% conditioned medium). Cell counts for embryos that did not hatch were not significantly different ($p > 0.05$) for the two groups, although they tended to be higher in fresh coculture than in conditioned medium (68 ± 10 cells vs. 48 ± 8, respectively).

DISCUSSION

This paper reports the superior ($p < 0.05$) development of bovine zygotes and early embryos in simple CZB medium with bovine oviduct epithelial cell coculture compared to embryos in coculture with complex serum-containing media. These data also demonstrate that monolayers from frozen-thawed BOEC and monolayer-conditioned medium can be used to develop bovine embryos past the 8–16-cell stage. However, embryonic development as assessed by total cell number, embryo quality score, and percentage of poor-quality nuclei, was significantly ($p < 0.05$) better in coculture with fresh BOEC monolayers. A significant ($p < 0.01$) inhibitory effect of glucose in the CZB medium on subsequent embryo blastulation during the first 36–48 h of embryo culture is also reported. Until now, glucose has been used in most media for bovine embryo culture and has been assumed to be useful.

In Experiment 1a, FBS-supplemented CMRL-1066 and Ham's F-10 were chosen to represent chemically complex serum-containing media. CMRL-1066 has been used extensively in a variety of cell culture systems including ones for primate and mouse embryos (Pope et al., 1982; Bates et al., 1985); improved differentiation and secretory function of endometrial cells and alveolar epithelium in CMRL versus Ham's F-10 has been reported (Doucet et al., 1987; Holinka and Gurpide, 1987). Ham's F-10 was chosen because of its extensive application in bovine embryo culture work (Wright and Bondoli, 1981; Rexroad, 1989).

CZB medium was chosen for its chemically simple nature and the encouraging results reported with its use in culturing embryos of other species (Chatot et al., 1989). Bovine serum albumin is somewhat heterogeneous, but it is

TABLE 5. Blastocyst formation in BOEC and embryo coculture (Experiment 2).

Coculture system	Medium: CZB + glucose — % of total embryos forming blastocysts	CZB without glucose — % of total embryos forming blastocysts	Treatment mean, %
Conditioned medium	14[a]	24[a]	19
Frozen-thawed coculture	9[a]	32[b]	20
Fresh coculture	17[a]	39[b]	28
Overall %	13[a]	31[b]	

[ab]Means in the same row with different superscripts differ at $p < 0.01$.

the only component in the CZB culture medium not chemically defined. The lack of FBS in this medium will facilitate biochemical analyses of BOEC secretions and their effects on embryos, since the major source of background protein is not present.

Energy substrates among the three media vary greatly. One disadvantage of supplementation with FBS is its inherent and variable energy sources. It is not possible to know with any degree of accuracy which energy sources are present in a given batch of serum-containing medium, although glucose surely is one. The CZB medium has the advantage that the energy sources are much better defined. The CZB medium is a modification of Brinster's BMOC-2 medium (Brinster, 1971), containing sodium pyruvate, sodium lactate, and glutamine. Glucose and serum are not present in CZB medium.

Neither CMFBS, HFFBS, nor CZB medium alone was able to support development of bovine 1-cell embryos past the 8–16-cell in vitro culture block. In Experiment 1a (Table 1) all three media with oviduct cell coculture supported development of embryos past the 16-cell stage (≥73%). With this clear-cut response to coculture in Experiment 1a, it seemed likely that the BOEC was producing material(s) beneficial to the young embryos and/or removing substances with negative effects from the media (Bavister, 1988).

The CZB with glucose and ITS/EGF added at 36–48 h was superior to CMFBS with added ITS/EGF in terms of cell number per embryo (Table 2). This suggested that CMFBS contained components not needed by the embryo and/or that some component or balance of components was slightly inhibitory. Because the lack of glucose in the CZB medium during the first 36 h could have been one of the factors responsible for these beneficial effects on development, a direct comparison was made in Experiment 2.

In Experiment 2, CZB without glucose initially and CZB with glucose throughout gave equivalent results in the quantity and quality of cells comprising the embryo up to the blastocyst stage. However, for blastocyst formation, CZB without initial glucose resulted in an average of 31% of the embryos forming blastocysts compared with an average of 13% for the three culture conditions when glucose was present throughout. The embryos in this study were cultured until Day 6.5 of the donor's cycle (estrus = Day 0). Lindner and Wright (1983) described a rapid progression of superovulated bovine embryos in vivo, with 8% having formed blastocysts on Day 6 and 60% on Day 7. The 31% blastocysts formed in this study by Day 6.5 in CZB without glucose seems similar to that found in vivo. In Experiment 3, 50% of the embryos cocultured with oviduct cells in CZB had formed blastocysts on Day 7. It is interesting to note that embryos cocultured with glucose throughout had cell numbers equal to those cultured without glucose during the first 36–48 h, but that many of these embryos failed to form a blastocoele cavity even with more than 100 cells. Therefore, the effect of glucose during the first 36–48 h of culture on blastocoele formation was latent, resulting from some unobserved earlier effect. Borland et al. (1976) described the role of active Na^+ and Cl^- transport for movement of fluid into the blastocoele cavity to form rabbit blastocysts. Although similar evaluations have not been done in cattle, one could hypothesize an early inhibition of synthetic products necessary for trophoblast-cell transport of solutes and water in those embryos cocultured with glucose initially.

Experiment 2 also revealed a substantial benefit to using fresh oviduct cell monolayers on embryo development compared with both frozen-thawed oviduct cell monolayers and conditioned medium, as evidenced by the percentage of poor-quality nuclei, number of cells per embryo, and embryo quality score. This study clearly indicates that embryos in the fresh BOEC system in vitro outperform similar embryos cultured on frozen-thawed BOEC or in conditioned medium. The trend continued when embryos were cultured 10 days, and a greater percentage (although not significant, $p < 0.10$) hatched in fresh BOEC coculture than with conditioned medium. This is in contrast to the results of Eyestone and First (1989), who found both coculture and conditioned media to give nearly similar results with in vitro-fertilized bovine embryos. However, the BOEC used in their study were cell suspensions rather than monolayers and the in vitro-fertilized oocytes developed overall at a lower rate (≤35% of cleaving embryos past 16 cells) than the in vivo-fertilized zygotes used in this study (≥85% of zygotes past 16 cells).

Other researchers found that conditioned medium did not support embryonic development as well as coculture in pigs (Allen and Wright, 1984) and sheep (Rexroad and Powell, 1988). The inability of conditioned medium to perform similarly to BOEC coculture may be an important clue to the mechanism of embryo development in coculture systems. It could be hypothesized that embryotrophic factors from the BOEC are less abundant in conditioned medium or that lack of monolayer cells fails to adequately remove factors from the embryonic microenvironment that inhibit development.

Rexroad and Powell (1988) reported the use of frozen-thawed oviduct cells for sheep embryo coculture. In contrast to our results, they found no difference in embryonic cleavage rates between fresh or frozen cell monolayers or Ham's F-10 medium alone after 5 days in culture. However, embryos cocultured with fresh or thawed oviduct cells did tend to have equally higher cleavage rates after transfer to recipients for three days than embryos cultured in media alone.

Embryos developed in CZB with fresh BOEC coculture have resulted in survival after transfer in our lab at rates (57%) similar to those for nonsurgical transfer of freshly recovered Day 7 bovine embryos (Ellington et al., 1990b).

In conclusion, early bovine embryos cocultured in simple serum-free CZB medium with fresh bovine oviduct epithelial cell monolayers had superior development to

embryos cocultured with similar monolayers in complex serum-containing media. Fresh oviduct cell monolayers also supported superior embryonic development in CZB medium compared to that in conditioned CZB medium or frozen-thawed cell monolayers. Furthermore, the presence of glucose in the CZB medium the first 36–48 h of embryo coculture resulted in significantly fewer embryos forming blastocysts at the end of Day 5.5 of culture. The CZB medium used in conjunction with bovine oviduct epithelial cell monolayers for in vitro culture allowed over 85% of the 1–2-cell bovine embryos to develop past 8–16-cells, and 33% of these embryos hatched in vitro after Day 10 in culture. The results of these studies indicate that it is now possible to develop early bovine embryos successfully in a simple coculture medium without glucose or serum.

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