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Early Embryonic Development in Vitro by Coculture with Oviductal Epithelial Cells in Pigs[1]

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ABSTRACT

This experiment was designed to evaluate the ability of three different somatic cell cultures to promote development of early cleavage stage pig embryos. A total of 245 2-cell, 4-cell, 8-cell, and 16-cell pig embryos were cocultured for 5 days with porcine oviductal epithelial cells (POEC), porcine fetal fibroblast monolayer (PEF), a combined POEC and PEF coculture system (PEF-POEC), or Dulbecco's Modified Eagle Medium alone (DMEM). Embryos were collected at slaughter from the reproductive tracts of superovulated prepubertal gilts. Embryos were recovered, evaluated, and randomly placed in one of the four treatment groups. POEC were recovered from oviductal flushes, washed, and placed in 24-well plates. PEF were obtained from 30-day to 60-day fetuses and established in culture. Finally, PEF-POEC consisted of a confluent monolayer of PEF in the bottom of 24-well plates also containing a Costar semipermeable membrane chamber with POEC in it. Embryos were evaluated every 24 h to determine stage of development. More ($p<0.05$) embryos developed to blastocysts in POEC (70% and 54%, respectively) and PEF-POEC (67% and 61%, respectively), than in either DMEM (16% and 2%, respectively) or PEF (27% and 23%, respectively). However, development of embryos did not differ ($p<0.05$) for POEC and PEF-POEC. These data indicate the presence of a primary culture of POEC promotes in vitro development of early cleavage stage pig embryos.

INTRODUCTION

Culture of early preimplantation mammalian embryos is currently an uncertain process. Pig embryos consistently fail to develop past the 4-cell stage. One assessment of in vitro viability is the ability of the culture to support development to the blastocyst and subsequent hatched blastocyst stages. Early cleavage stage embryos are usually transferred to an intermediate host for culture to facilitate embryonic development. These embryos are then recovered 3–4 days later and transferred to an appropriate recipient for development to term. However, this technique limits the ability to manipulate early embryos and study embryological development. In addition, this technique is laborious and expensive. Currently, data indicate that coculture with fetal fibroblast or oviductal monolayers appears to contribute to an increase in embryonic development in ovine and bovine preimplantation embryos (Rexroad and Powell, 1986; Kuzan and Wright, 1982a). Results from Gandolfi and Moore (1987) indicate potential differences in both the type of cells used as well as the state (nonestablished) of cells during coculture. These data indicate differences in embryonic development during in vitro culture (>3 days) and subsequent in vivo survival after culture between embryos cocultured with established fibroblast cells (5% and 46%) and those cocultured with nonestablished sheep oviductal epithelial cells (33% and 80%). Therefore, the purpose of this experiment was to determine the ability of porcine oviductal epithelial cells to facilitate in vitro development of early cleavage stage porcine embryos when compared to medium alone or to an established cell line (porcine fetal fibroblasts), as well as possible synergy between cell types to promote development.

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MATERIALS AND METHODS

Cell Cultures

We evaluated three different somatic cell cultures to determine their ability to promote development of early cleavage stage porcine embryos. Porcine fetal endometrial fibroblast (PEF) monolayers were obtained from 30–60 day female fetuses. Fetuses were obtained at slaughter and removed from the reproductive tract aseptically; they were sexed and the female reproductive tracts were removed. Tracts were then cut into 1–2 mm^2 explants, washed with Dulbecco's Modified Eagle Medium (DMEM) containing 10% (v/V) fetal calf serum (Hyclone Laboratories, Inc., Logan, UT) (DMEM-1) and 5% (V/V) antibiotic/antimycotic (DMEM-2) and placed in 25-cm^2 culture flasks (Costar, Cambridge, MA). Explants were initially cultured in DMEM-1 containing 2% (V/V) antibiotic/antimycotic; however, levels of antibiotic/antimycotic were slowly decreased with each subsequent passage until all cultures were maintained with DMEM-1. Cells were established in vitro and subcultured a minimum of ten passages prior to use in these experiments. PEF were transferred to wells of a 24-well tissue culture plate 2 days prior to embryo culture and monolayers were developed to 70–80% confluence prior to initiation of embryo culture. Prior (2 h) to embryo culture, medium was changed and replaced with DMEM containing 10% (V/V) fetal calf serum and 5 mg/ml bovine serum albumin (Sigma Chemical Co., St. Louis, MO) (DMEM-3).

Porcine oviductal epithelial cells (POEC) were recovered at embryo collection from the oviductal flushes of Day-1 and Day-2 (Day 0 = ovulation) embryo donors. Embryos were immediately removed from flushes and remaining cells were centrifuged at 250 × *g* for 5 min. The supernatant was discarded and cells were resuspended in DMEM-2. After an additional wash, cells were pooled, and resuspended in DMEM-3; cell number was determined by use of a hemacytometer. A total of 1–2 × 10^5 cells was placed in wells of a 24-well tissue culture plate and incubated for 2 h prior to embryo culture. POEC obtained by this technique result in a high proportion of cells with functional cilia and superior morphology.

PEF-POEC culture consisted of a 70–80% confluent PEF monolayer in wells of a 24-well tissue culture plate. Each well also contained a transwell membrane (Costar) with pore size of 0.4 μm. After POEC were collected and washed, 1–2 × 10^5 cells were placed in the transwell and incubated 2 h prior to embryo culture. This preincubation was carried out to equilibrate medium (DMEM-3) between the 0.4-μm membrane prior to initiation of embryo culture. Embryos were subsequently placed in the transwell (POEC side) for culture.

Embryo Collections

Embryos were recovered at slaughter from the reproductive tracts of 27 crossbred prepubertal gilts that had received 400 IU pregnant mare's serum gonadotropin (PMSG) (Diosynth, Chicago, IL) and 200 IU human chorionic gonadotropin (hCG) (Organon, West Orange, NJ) followed 78 h later by 500 IU hCG. All animals were artificially inseminated 32 h after final hCG injection. Embryos were collected 1–3 days after ovulation. Day-1 and Day-2 (2-cell to 4-cell) embryos were obtained by flushing 60–100 ml phosphate-buffered saline (PBS) supplemented with 1% fetal calf serum (PBS-1) from the ostium of the oviduct through the oviduct and out a cannula placed into the oviduct through the utero-tubal junction. Day-3 (8-cell to 16-cell) embryos were collected by flushing 200–240 ml PBS-1 from the ostium of the oviduct through the oviduct and out a cannula placed 15–30 cm distal to the utero-tubal junction. Flush medium from each of the two groups of donors (Day-1 to Day-2 and Day-3) were collected in separate sterile bottles; embryos were identified by use of a dissecting microscope, washed, and placed in fresh PBS-1. Oviductal cells recovered in flushes of Day-1 and Day-2 donors were prepared and used in coculture as previously described. Embryos were pooled from all donors to remove any potential donor effects and evaluated for both stage of development and quality.

Embryo Culture

Embryos were randomly assigned by stage and quality to each of the four culture groups (PEF, POEC, PEF-POEC, and DMEM). All embryo cultures were carried out in DMEM-3 at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Embryos were removed from culture and evaluated every 24 h with respect to stage of development. Evaluations were made by use of a Ziess axiovert 405 microscope at a 100–200× magnification. In vitro viability was determined by the ability of culture to support development to the hatched blastocyst stage. Embryos were cultured for up to 5 days in vitro and the number at each stage within groups was recorded. All embryos observed at the 2-cell stage were considered

TABLE 1. In vitro development of pig embryos after culture in various culture systems.

Treatment	Initial stage				Stage of development	
	2	4	8	16	Blastocyst	Hatched blastocyst
PEF	12	14	13	21	16 (27%)[a]	14 (23%)[a]
POEC	13	14	14	22	44 (70%)[b]	34 (54%)[b]
PEF-POEC	12	15	12	22	41 (67%)[b]	37 (61%)[b]
DMEM	11	15	14	21	10 (16%)[a]	1 (2%)[a]

[ab]Data in columns with different superscripts are different ($p<0.05$).

fertilized and included in the experiment. One-cell embryos were not included in the experiment to eliminate the potential introduction of unfertilized eggs into treatment groups (comprised <12%, 29/245, of total embryos collected). Although staining techniques are available to evaluate 1-cell embryos for fertilization, viability of these cells after staining is not clear. In addition, previous data (Davis and Day, 1978; Wright and Bondioli, 1981) in pigs indicate no advantages to initiating culture at the 1-cell stage, compared to the 2-cell stage, with respect to in vitro developmental capacity (i.e. no difference between percentage of 1-cell and 2-cell porcine embryos that develop past the 4-cell block).

Statistical Analysis

Data were analyzed by chi-square analysis to determine differences between groups.

RESULTS

Totals of 60, 63, 61, and 61 embryos were cocultured in PEF, POEC, PEF-POEC, and DMEM-3, respectively (Table 1). Of the embryos cultured with PEF, POEC, and PEF-POEC, 16 (27%), 44 (70%), and 41 (67%) developed to the blastocyst stage; and 14 (23%), 34 (54%), and 37 (61%) subsequently developed into hatched blastocysts, respectively. A total of 38%, 11%, 7%, and 77% of 2-cell and 4-cell embryos arrested after 1–2 cleavages when cultured in PEF, POEC, PEF-POEC, and DMEM, respectively.

When embryonic development was evaluated with respect to stage of development at initiation of culture, 25%, 62%, and 67% of 2-cell; 7%, 57%, and 53% of 4-cell; 31%, 71%, and 75% of 8-cell; and 38%, 82%, and 73% of 16-cell embryos developed to the blastocyst stage in PEF, POEC, and PEF-POEC groups compared to 0%, 0%, 7%, and 43% in DMEM, respectively (Fig. 1). Both POEC and PEF-POEC coculture groups were different ($p<0.05$) from other (PEF and DMEM) treatments at all stages evaluated. There was no difference ($p>0.05$) in subsequent development of embryos to the blastocyst or hatched blastocyst stages in either POEC or PEF-POEC, regardless of initial embryonic stage cultured. In addition, there were significant differences ($p<0.05$) in the ability of these culture systems to promote development to the hatched blastocyst stage. A larger number of embryos developed to the hatched blastocyst stage in both POEC and PEF-POEC (46% and 58%, 2-cell; 36% and 53%, 4-cell; 50% and 58%, 8-cell; 73% and 68%, 16-cell, respectively) compared to PEF or DMEM culture groups (17% and 0%, 2-cell; 0% and 0%, 4-cell; 31% and 0%, 8-cell; 38% and 5%, 16-cell, respectively) regardless of stage at initiation of culture (Fig. 2).

DISCUSSION

Most in vitro culture systems are unable to maintain development of embryos from early cell stages through hatching. The majority of these embryos stop developing after one to two cleavages due to an apparent inadequacy of culture systems used. However, if embryos are transferred to the environment of the oviduct, development from 1-cell to blastocyst is possible. Data indicate a high percentage of 1-cell bovine embryos cultured in this manner in both sheep (Leibfried-Rutledge et al., 1987) and rabbit (Boland et al., 1984) oviducts develop to the blastocyst stage. These results appear to indicate that the beneficial effects of oviducts on embryonic development are not species-specific. Early studies using hormonally primed explanted mouse oviducts in coculture with mouse embryos reported facilitated development of 1-cell to 2-cell mouse embryos through the block (Biggers et al., 1961; Whittingham, 1968). In consideration of the data reported here, primary cultures of oviductal epithelial cells in coculture may provide a means to successfully culture early embryos of other domestic animals. In addition, by

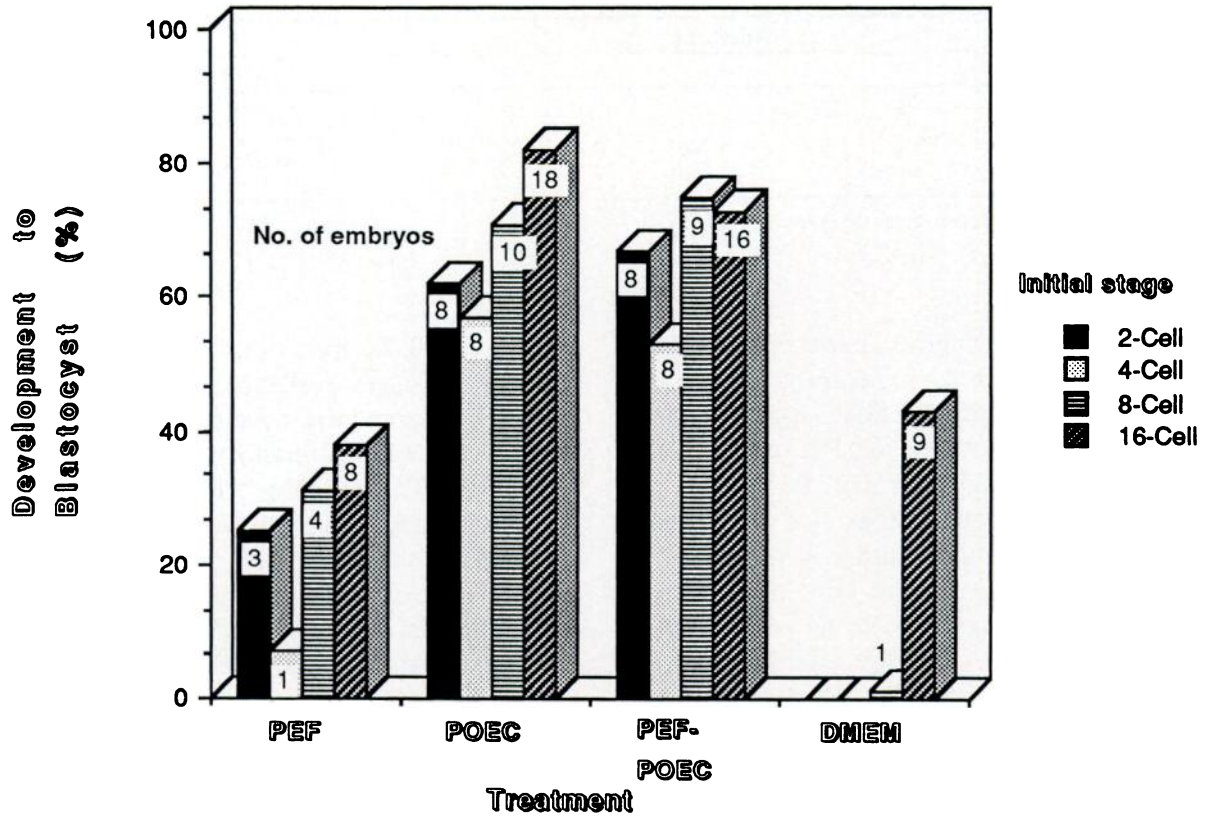

FIG. 1. Percent embryonic development to the blastocyst stage after culture in vitro in each of the treatment groups; PEF, POEC, PEF-POEC, and DMEM. Embryos were cultured at 37°C in a humidified atmosphere of 5% CO_2 and air and evaluated every 24 h to assess developmental stage and quality. Data are represented with respect to stage of development at initiation of culture (2-cell, 4-cell, 8-cell, or 16-cell), and total number of embryos that reached the blastocyst stage is indicated with each individual bar. A significantly ($p<0.05$) greater percentage of embryos developed to the blastocyst stage in both the POEC and PEF-POEC groups compared to the other groups, regardless of developmental stage at initiation of culture.

using coculture in conjunction with human in vitro fertilization systems, increased embryonic development may also be achieved.

The tremendous success of Gandolfi and Moor (1987) with respect to culture of sheep embryos with oviduct cells indicates the ability of these cells to support "normal" development in vitro. Transfer of these embryos following in vitro culture resulted in a high percentage of embryos continuing to develop in vivo. These results establish this technique as superior to other successful culture techniques involving medium alone or coculture with other cell types such as trophoblastic vesicles (Heyman et al., 1987), fibroblast monolayers (Kuzan and Wright, 1982b), or chicken amniotic fluid (Blakewood et al., 1988).

Results from experiments reported here indicate early in vitro development of porcine embryos is facilitated by coculture with porcine oviductal epithelial cells. These data compare favorably with the data of Gandolfi and Moore (1987) concerning in vitro development. To our knowledge, this coculture system represents the most efficient in vitro porcine embryo culture system reported, with respect to early stage embryos.

It has been suggested that there may be at least two components of an in vitro culture system: 1) the ability to support development through the transitional (block) stage to the blastocyst stage, and 2) the ability to facilitate the acquisition of full embryonic viability (Gandolfi and Moore, 1987). This may be accomplished in vitro and relate to either the presence of a stimulatory component or removal of inhibitory components (Bavister, 1988) within normal in vitro culture media or a combination of both components. Results of this experiment support this hypothesis with respect to facilitation of development through the block.

The in vitro block appears to be related to embryonic genome activation, increased DNA synthesis, both qualitative and quantitative changes in amino acid uptake,

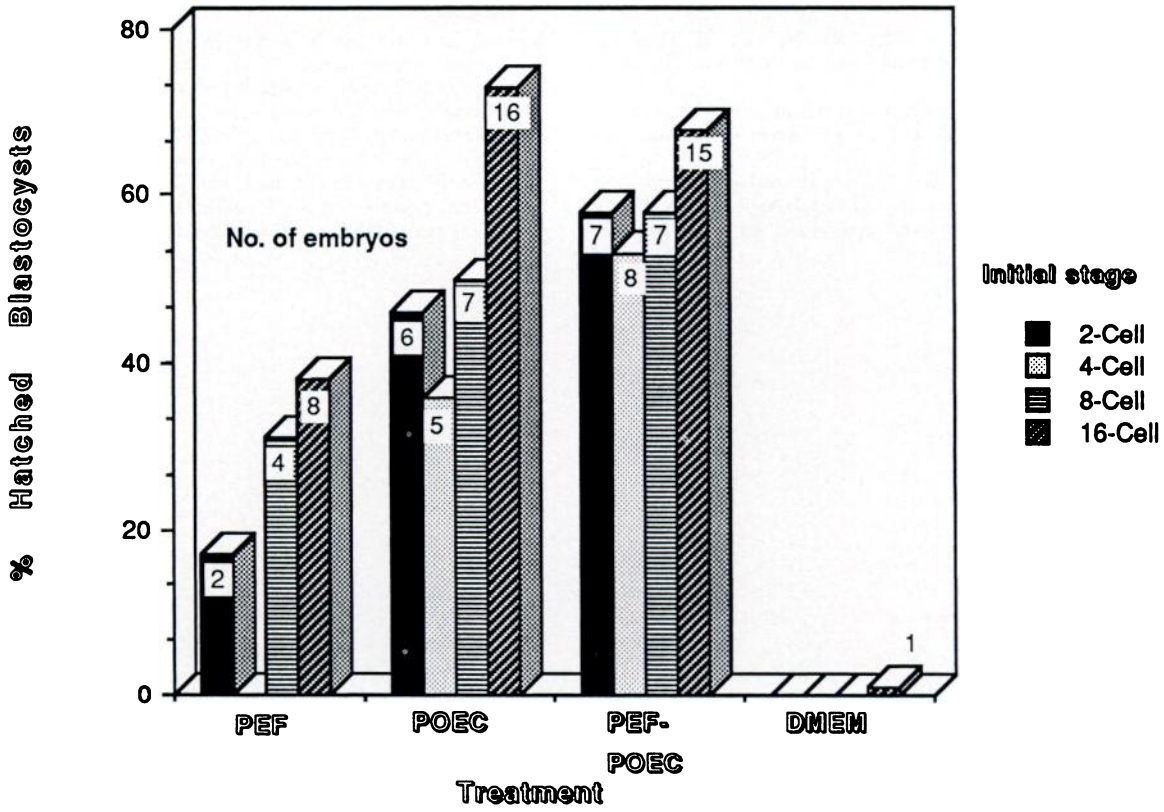

FIG. 2. Percent embryonic development to the hatched blastocyst stage after culture in vitro in each of the treatment groups: PEF, POEC, PEF-POEC, and DMEM. Embryos were cultured at 37°C in a humidified atmosphere of 5% CO_2 and air and evaluated every 24 h, to assess developmental stage and quality. Data are represented with respect to stage of development at initiation of culture (2-cell, 4-cell, 8-cell, or 16-cell), and total number of embryos that reached the blastocyst stage is indicated with each individual bar. A total of 46, 36, 50, and 73% of embryos cocultured with POEC and 58, 53, 58, and 68% of embryos cocultured with PEF-POEC developed to the hatched blastocyst stage after in vitro culture when initiated at the 2-cell, 4-cell, 8-cell, and 16-cell stages, respectively. This was significantly different ($p<0.05$) from all other culture groups.

and morphological maturation of organelles (Minami et al., 1988). In vitro developmental blocks have been reported at the 2-cell stage in mice, hamsters, and gerbils (Goddard and Pratt, 1983; Whittingham and Bavister, 1974; Norris et al., 1985), 8-cell stage in sheep (Gandolfi and Moore, 1987), and 4-cell stage in pigs (Polge, 1982; Davis, 1985). Our results indicate POEC provide factors necessary for cleavage of pig embryos through the 4-cell block (only 11% and 7% of embryos cultured in POEC and PEF-POEC failed to develop past the block) and subsequently to the hatched blastocyst stage. In addition, nonestablished oviductal cells may promote in vitro development of porcine embryos differently than established fibroblast cell lines. This appears to result either from more efficient removal of inhibitory components or production of factors required for development through the transitional stage and subsequent normal embryonic viability.

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