Comparison of the effects of oviductal cell co-culture and oviductal cell-conditioned medium on the development and metabolic activity of cattle embryos

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The objective of this study was to compare the development and metabolic activity of cattle embryos co-cultured with bovine oviductal cells or cultured in serum-free medium previously conditioned by bovine oviductal cells. Zygotes were produced by *in vitro* fertilization of oocytes from bovine ovaries obtained from an abattoir. Development to the four-cell stage occurred by 48 h after fertilization in both culture systems, but co-cultured embryos reached the 16-cell stage by 96 h, whereas those cultured in conditioned medium did not do so until 24 h later. Similarly, the morula and blastocyst stages were reached 24 h earlier in co-culture than in conditioned medium. There were significantly more cells in the blastocysts from co-culture (96.8 ± 6.1 versus 56.7 ± 3.3 ; $P \leq 0.0001$). The metabolism of glutamine did not differ between embryos cultured in the two systems, but the metabolism of glucose was significantly greater in embryos cultured in conditioned medium. The first significant increase in glucose metabolism occurred between the four-cell and the 16-cell stages in embryos cultured in conditioned medium, but occurred between the 16-cell and morula stages in the co-cultured embryos, such that the glucose metabolism was significantly greater at the 16-cell stage in embryos cultured in conditioned medium compared with co-cultured embryos (6.5 ± 1.0 versus 1.5 ± 0.4 pmol per embryo per 3 h, $P \leq 0.0001$). The concentration of glucose was significantly less, and that of lactate significantly greater, in co-culture medium than in conditioned medium. The results suggest that the activity of enzymes involved in glucose transport or metabolism in the early cattle embryo can be affected by the prior culture conditions, and that a high rate of glucose metabolism may be unfavourable for development.

Introduction

In general, the development of cattle embryos is arrested or blocked between the eight-cell and 16-cell stages when they are cultured in conventional complex cell-culture media (reviewed by Thibault, 1966; First and Parrish, 1987; Greve and Madison, 1991). Co-culture with oviductal cells, or culture in oviductal cell-conditioned medium, can support the development of sheep (Gandolfi and Moor, 1987; Rexroad and Powell, 1988) and cattle (Fukui and Ono, 1988; Eyestone and First, 1989; Ellington *et al.*, 1990; Gordon and Lu, 1990; Mermillod *et al.*, 1992a) embryos through the block.

We have shown that cattle embryos produced by *in vitro* maturation and fertilization of oocytes collected from ovaries obtained from an abattoir develop to the blastocyst stage, expand, and hatch in culture, and produce pregnancies and live calves after transfer to recipient cows after either co-culture

with bovine oviductal cells (Xu *et al.*, 1992a, b), or culture in medium that has been conditioned with bovine oviductal cells (Mermillod *et al.*, 1992a, b). Calves have also been produced from such embryos after micromanipulation (Loskutoff *et al.*, 1993; Johnson *et al.*, 1995) and cryopreservation (Massip *et al.*, 1993; J. W. Pollard and S. P. Leibo, personal communication).

However, there is a significant difference in the timing of development in the two culture systems. Development to the four-cell stage occurs at 40–48 h after *in vitro* fertilization in both co-culture and in conditioned medium, whereas development to the 16-cell stage occurs by, on average, 90 h in co-culture (Rieger *et al.*, 1992a), but it does not occur until 120 h in conditioned medium, owing to an extended period at the 5–8-cell stage (Grisart *et al.*, 1994). This delay in development in conditioned medium is of interest because it is intermediate between the total arrest seen in conventional complex cell-culture media and the fast development seen in co-culture. It therefore offers a model to investigate the mechanism(s) by which somatic cells support the development

of embryos *in vitro*. The difference in timing of development is also of interest because there is a significant change in the patterns of energy metabolism of co-cultured cattle embryos between the four-cell and blastocyst stages (Rieger *et al.*, 1992a, b).

The objective of the present experiments was to compare directly the effects of co-culture and culture in conditioned medium on the development of cattle embryos, and to determine whether the differences in development were accompanied by differences in metabolic activity. The production of $^3\text{H}_2\text{O}$ from D-[5- ^3H]glucose was used as a measure of total anaerobic glucose metabolism (to phosphoenolpyruvate), and the production of $^{14}\text{CO}_2$ from L-[^{14}C (U)]glutamine by the Krebs cycle was used as a measure of mitochondrial oxidative metabolism (Rieger and Guay, 1988; Rieger *et al.*, 1992a, b).

Materials and Methods

Culture reagents

Fetal calf serum (FCS), TCM-199, penicillin and streptomycin were obtained from Gibco BRL (Paisley). Porcine FSH (pFSH) and LH (pLH) were kindly provided by J. F. Beckers, Liège. Heat-treated donor steer serum was purchased from Cansera, Inc., (Rexdale, Ontario), paraffin oil from Merck (Darmstadt), and silicone oil from Dow Corning (Midland, MI). Unless otherwise indicated, all other culture reagents were "cell culture tested" reagents from Sigma (St Louis, MO).

Oocyte collection, *in vitro* maturation and *in vitro* fertilization

The experiments were conducted in Louvain-la-Neuve. Four separate batches of oocyte-cumulus complexes ($n = 369, 218, 258$ and 268) were matured and fertilized *in vitro* using methods slightly modified from those described by Mermillod *et al.* (1993). Oocyte-cumulus complexes were collected by aspiration of 2–5 mm follicles from cow ovaries obtained from an abattoir, and washed three times in modified Dulbecco's PBS containing $3.3 \text{ mmol pyruvate l}^{-1}$ and $4 \text{ mg fraction V BSA ml}^{-1}$ (Buffer A). Groups of 100 oocyte-cumulus complexes were matured in $500 \mu\text{l}$ maturation medium (TCM-199 containing 10% (v/v) fetal calf serum, $1 \mu\text{g oestradiol ml}^{-1}$, $5 \mu\text{g pLH ml}^{-1}$ and $0.5 \mu\text{g pFSH ml}^{-1}$). After maturation for 24 h at 39°C in a humidified atmosphere of 5% CO_2 in air, the oocytes were washed three times in Buffer A and transferred into $500 \mu\text{l}$ fertilization medium (Tyrode's medium containing albumin, lactate and pyruvate (TALP) supplemented with $10 \mu\text{g heparin ml}^{-1}$).

Spermatozoa were selected by centrifugation of thawed semen on a Percoll discontinuous density gradient (45/90%) for 30 min at 700 g . After a second centrifugation in TALP to remove any traces of Percoll, the living spermatozoa were added to the fertilization well to obtain a final concentration of $2 \times 10^6 \text{ ml}^{-1}$. After fertilization for 18 h at 39°C , the presumptive zygotes were stripped of cumulus cells by vortexing for 2 min, and washed twice in Buffer A and twice in culture medium.

Approximately equal numbers of the presumptive zygotes (all the oocytes, without selection) were then assigned

randomly to two culture treatment groups: co-culture with bovine oviductal epithelial cells, or culture in medium conditioned by bovine oviductal epithelial cells.

Embryo co-culture with bovine oviductal epithelial cells

The medium used for co-culture was TCM-199 supplemented with $60\,000 \text{ iu penicillin l}^{-1}$ and $60 \text{ mg streptomycin l}^{-1}$, and with $5 \text{ mmol sodium pyruvate l}^{-1}$ and $2 \text{ mmol L-glutamine l}^{-1}$. Osmolality was adjusted to 280 mosm l^{-1} with sterile distilled water before addition of 10% (v/v) heat-treated donor steer serum and 3.5 g BSA l^{-1} (Fraction V).

On the same day as oocyte collection, an oviduct was processed to produce oviductal cell cylinders as described by Xu *et al.* (1992b). On the next day, groups of 40–50 oviductal cell cylinders were placed in $50 \mu\text{l}$ drops of co-culture medium under silicone oil (Dow Corning, Midland, MI) and cultured at 39°C in an atmosphere of 5% CO_2 in air for 24 h. Groups of 30 presumptive zygotes were then added to each culture drop (at 18 h after insemination) and cultured for up to 7 days. At 48 h after the zygotes were introduced, each co-culture drop was supplemented with $25 \mu\text{l}$ serum-free co-culture medium. At 120 h, each co-culture drop was supplemented with $25 \mu\text{l}$ serum-replete co-culture medium.

Embryo culture in serum-free medium conditioned by bovine oviductal epithelial cells

Cow oviducts were randomly obtained from an abattoir. Bovine oviductal epithelial cells were isolated and cultured to produce monolayers, as described by Eyestone and First (1989). Embryo culture medium was conditioned by these monolayers under serum-free conditions and harvested and stored until required according to the procedure detailed by Mermillod *et al.* (1993). At 18 h after insemination, groups of 40 presumptive zygotes were placed in $40 \mu\text{l}$ drops of conditioned medium under paraffin oil and cultured at 39°C in a humidified atmosphere of 5% CO_2 in air, for up to 8 days.

Metabolic measurements

The medium used for the metabolic measurements was a modified synthetic oviductal fluid (Tervit *et al.*, 1972) containing $108 \text{ mmol NaCl l}^{-1}$, $25 \text{ mmol NaHCO}_3 \text{ l}^{-1}$, $7.2 \text{ mmol KCl l}^{-1}$, $1.7 \text{ mmol CaCl}_2 \text{ l}^{-1}$, $1.2 \text{ mmol KH}_2\text{PO}_4 \text{ l}^{-1}$, $0.5 \text{ mmol MgCl}_2 \text{ l}^{-1}$, $1.0 \text{ mmol glucose l}^{-1}$, $1.0 \text{ mmol pyruvate l}^{-1}$, $1.5 \text{ mmol L-glutamine l}^{-1}$, $3.5 \text{ g essential fatty acid-free bovine serum albumin l}^{-1}$, $100\,000 \text{ iu penicillin l}^{-1}$, $100 \text{ mg streptomycin l}^{-1}$, and $20 \text{ mmol Hepes l}^{-1}$.

Groups of 8–12 embryos at the four-cell, nominal 16-cell, compacted morula, and blastocyst stages were removed from co-culture at 48, 96, 144 and 168 h after insemination, and from conditioned medium culture at 48, 120, 168 and 192 h after insemination, respectively. Each of the four developmental stages was derived from a separate batch of oocytes. At each stage, the co-cultured embryos and those cultured in conditioned medium were derived from the same batch of oocytes. The embryos were washed four times and cultured for

4 h in metabolic measurement medium at 39°C in a humidified atmosphere of 5% CO₂ in air before being subjected to the metabolic measurement procedure.

Except for the medium, the procedure used to measure embryo metabolism was exactly as described by Rieger *et al.* (1992a). A mixture of L-[U-¹⁴C]glutamine (specific activity = 262 mCi mmol⁻¹, NEN, Dupont, Wilmington, DE) and D-[5-³H]glucose (specific activity = 15.7 Ci mmol⁻¹, Amersham, Little Chalfont) was dried under nitrogen and taken up in metabolic measurement medium to give nominal concentrations of 0.25 µCi µl⁻¹ for each labelled substrate. The final concentrations (labelled plus unlabelled) were 2.0 mmol l⁻¹ and 1.0 mmol l⁻¹ for glutamine and glucose, respectively. The amount of glutamine and glucose metabolized by each embryo was calculated as described by Tiffin *et al.* (1991). The sensitivities of the assays were 0.6 pmol per 3 h for glutamine, and 0.1 pmol per 3 h for glucose.

Cell counting

Immediately after the metabolic measurement procedure, the embryos were fixed with 70% (v/v) ethanol using the procedure described by Mermillod *et al.* (1993). After ethanol fixation, their nuclei were stained using Hoechst-33342 dye as described by Pursel *et al.* (1985) and counted under an epifluorescence microscope (Nikon, Optiphot) equipped with an excitation filter of 330–380 nm and a barrier filter of 420 nm.

Measurement of glucose and lactate concentrations in co-culture medium and in conditioned medium

In a separate experiment, 50 µl drops containing bovine oviductal epithelial cells were prepared and cultured for 7 days, as described above for co-culture, but without embryos. Time 0 was defined as the time when embryos were introduced into the culture drops. In two trials, samples of medium were taken at -24, 0, 24, 48, 120 and 144 h. At 48 and 120 h, samples were taken immediately before, and immediately after, the supplementation with fresh medium. Samples were also taken from six batches of conditioned medium that were prepared as described above.

The glucose concentrations in 100 µl aliquots of the media samples were determined by spectrophotometric measurement of the reduction of nicotinamide adenine dinucleotide by a hexokinase–glucose-6-phosphate dehydrogenase-coupled reaction (Coulter glucose reagent, Coulter Electronics, Burlington, Ontario).

The lactate concentrations in 10 µl aliquots of the media samples were determined by colorimetric measurement of the red peroxidation product of 4-aminoantipyrine and 1,7-dihydroxynaphthalene formed by a lactate oxidase–horseradish peroxidase coupled reaction (Kodak Ektachem DT Slide (LAC), Kodak Clinical Products, Rochester, NY).

Statistical analysis

The numbers of cells at the nominally 16-cell, morula and blastocyst stages produced in co-culture and conditioned

medium were compared by orthogonal contrasts. All other data were log-transformed, where $y' = \log(y + 1)$, to provide homogeneity of variance for statistical analysis using SuperANOVA (Abacus Concepts Inc., Berkeley, CA). The effects of culture treatment and stage of development on the measurements of metabolism were evaluated by two-way analyses of variance. For analyses with significant effects of stage of development and treatment × stage interaction, the means of successive stages within each treatment, and means of the same stage between treatments, were compared by orthogonal contrasts. For analyses with a significant effect of stage of development, but no effect of treatment × stage interaction, the pooled means of successive stages were compared by orthogonal contrasts. The effect of time on the concentrations of glucose and lactate in co-culture medium was evaluated by one-way analysis of variance for repeated measures. Mean concentrations in successive samples were compared by orthogonal contrasts.

Results

Comparison of embryo development in co-culture and in conditioned medium

The embryos reached the four-cell stage by 48 h after insemination in both co-culture and in conditioned medium (Table 1). One nominally 16-cell conditioned-medium embryo was lost after the metabolic measurement, and the numbers of cells in three conditioned-medium blastocysts could not be determined. The actual numbers of cells at the nominal 16-cell stage ranged from 11 to 19 in the co-cultured embryos and from 13 to 28 in the embryos cultured in conditioned medium, but the means were not different ($P > 0.05$). However, whereas the embryos in co-culture reached the 16-cell stage by 96 h, those cultured in conditioned medium did not do so until 120 h, 24 h later. Similarly, the morula and blastocyst stages were reached 24 h earlier in co-culture than in conditioned medium. The number of cells in the morulae produced in the two systems did not differ ($P > 0.05$). However, at the blastocyst stage, there were significantly more cells in the embryos from co-culture ($P \leq 0.0001$).

Comparison of glucose and glutamine metabolism by embryos co-cultured with oviductal cells or cultured in conditioned medium

Overall, the embryos cultured in conditioned medium metabolized significantly more glucose than did the co-cultured embryos ($P \leq 0.0001$). There were also highly significant effects of stage of development ($P \leq 0.0001$), and treatment × stage interaction ($P \leq 0.01$) on glucose metabolism (Table 2). For the co-cultured embryos, the first significant increase in glucose metabolism occurred between the 16-cell and morula stages, whilst the first significant increase occurred between the four-cell and 16-cell stages in the embryos cultured in conditioned medium. Glucose metabolism at the 16-cell stage was significantly greater in embryos cultured in conditioned medium than in co-cultured embryos.

Table 1. The times of recovery and numbers of cells in cattle embryos co-cultured with bovine oviductal epithelial cells or in conditioned medium

Stage	Culture treatment					
	Co-culture			Conditioned medium		
	Number of embryos	Time* (h)	Number of cells	Number of embryos	Time (h)	Number of cells
Four-cell	12	48	4	12	48	4
16-cell	7	96	15.4 ± 0.9	8	120	19.1 ± 1.9
Morula	12	144	30.5 ± 3.1	11	168	27.3 ± 3.8
Blastocyst	11	168	96.8 ± 6.1	9	192	56.7 ± 3.3 ^a

*Time after insemination.

Numbers of cells are means ± SEM.

^aSignificantly different from the same stage in the co-culture group ($P \leq 0.0001$).**Table 2.** The effect of co-culture with bovine oviductal epithelial cells or culture in conditioned medium, and stage of development, on the metabolism of glucose by cattle embryos

Stage	Culture treatment					
	Co-culture		Conditioned medium		Pooled	
	Number of embryos	Metabolism (pmol per embryo per 3 h)	Number of embryos	Metabolism (pmol per embryo per 3 h)	Number of embryos	Metabolism (pmol per embryo per 3 h)
Four-cell	12	0.7 ± 0.1	12	1.5 ± 0.6	24	1.1 ± 0.3
16-cell	7	1.5 ± 0.4	9	6.5 ± 1.0 ^{ab}	16	4.4 ± 0.9
Morula	12	17.7 ± 2.5 ^a	11	22.4 ± 3.1 ^a	23	19.9 ± 2.0
Blastocyst	11	35.0 ± 2.9 ^a	12	44.8 ± 5.0 ^a	23	40.1 ± 3.1

Metabolism values are means ± SEM.

^aSignificantly different from the preceding stage in the same column ($P \leq 0.0002$).^bSignificantly different from the same stage in the co-culture group ($P \leq 0.0001$).

The embryos cultured in conditioned medium also metabolized significantly more glucose per cell than did the co-cultured embryos ($P \leq 0.0001$). There was a highly significant effect of stage of development ($P \leq 0.0001$) on glucose metabolism per cell, but no effect of treatment × stage interaction (Table 3). For the pooled data, there was a highly significant increase between the 16-cell and morula stages, but no difference between the four-cell and 16-cell stages, or between the morula and blastocyst stages.

There was a highly significant effect of stage of development ($P \leq 0.0001$) on glutamine metabolism but no significant effect of culture treatment or interaction (Table 4). For the pooled data, glutamine metabolism tended to decrease from the four-cell to 16-cell stage ($P \leq 0.06$), and increased significantly ($P \leq 0.05$) between the 16-cell and morula stages and between the morula and blastocyst stages.

There was a highly significant effect of stage of development on glutamine metabolism per cell ($P \leq 0.0001$), but no effect of culture treatment or interaction (Table 5). For the pooled data, there was a highly significant decrease in glutamine metabolism per cell between the four-cell and 16-cell stages, but no change thereafter.

There were highly significant effects of culture treatment and stage of development ($P \leq 0.0001$), and of treatment × stage interaction ($P \leq 0.005$) on the ratio of glucose metabolism to glutamine metabolism (Table 6). In the co-cultured embryos, the metabolism ratio increased significantly between the 16-cell and morula stages, but was not different between the four-cell and 16-cell stages, nor between the morula and blastocyst stages. In the embryos cultured in conditioned medium, the ratio increased significantly between the four-cell and 16-cell stages, and again between the 16-cell and morula stages, but was unchanged between the morula and blastocyst stages. At the 16-cell stage, the ratio was significantly greater in the embryos cultured in conditioned medium than in the co-cultured embryos.

Glucose and lactate concentrations in co-culture medium and in conditioned medium

There was a significant effect of culture time on the concentrations of both glucose ($P \leq 0.0002$) and lactate ($P \leq 0.0001$) in the bovine oviductal epithelial cell co-culture

Table 3. The effect of co-culture with bovine oviductal epithelial cells or culture in conditioned medium, and stage of development, on glucose metabolism per cell by cattle embryos

Stage	Culture treatment					
	Co-culture		Conditioned medium		Pooled	
	Number of embryos	Metabolism (pmol per cell per 3 h)	Number of embryos	Metabolism (pmol per cell per 3 h)	Number of embryos	Metabolism (pmol per cell per 3 h)
Four-cell	12	0.2 ± 0.0	12	0.4 ± 0.1	24	0.3 ± 0.1
16-cell	7	0.1 ± 0.0	8	0.4 ± 0.0	15	0.2 ± 0.0
Morula	12	0.6 ± 0.1	11	0.9 ± 0.1	23	0.7 ± 0.1 ^a
Blastocyst	11	0.4 ± 0.0	9	0.8 ± 0.1	20	0.6 ± 0.1

Metabolism values are means ± SEM.

^aSignificantly different from the preceding mean in the same column ($P \leq 0.0001$).**Table 4.** The effect of co-culture with bovine oviductal epithelial cells or culture in conditioned medium, and stage of development, on the metabolism of glutamine by cattle embryos

Stage	Culture treatment					
	Co-culture		Conditioned medium		Pooled	
	Number of embryos	Metabolism (pmol per embryo per 3 h)	Number of embryos	Metabolism (pmol per embryo per 3 h)	Number of embryos	Metabolism (pmol per embryo per 3 h)
Four-cell	12	3.3 ± 0.5	12	4.3 ± 0.8	24	3.8 ± 0.5
16-cell	7	2.4 ± 0.5	9	2.7 ± 0.5	16	2.6 ± 0.4
Morula	12	4.2 ± 0.9	11	4.2 ± 0.6	23	4.2 ± 0.6 ^a
Blastocyst	11	7.4 ± 0.9	12	6.6 ± 0.6	23	7.0 ± 0.5 ^a

Metabolism values are means ± SEM.

^aSignificantly different from the preceding stage in the same column ($P \leq 0.05$).

medium (Fig. 1). Glucose fell from 5.8 mmol l^{-1} , when the epithelial cells were placed in the droplets at -24 h , to 0.6 mmol l^{-1} 24 h later, when embryos normally would be introduced (time 0). Glucose concentration remained low until 144 h , rising briefly after the drops were supplemented with fresh medium at 48 and 120 h . The pattern for lactate was exactly opposite to that of glucose, increasing from 1.2 mmol l^{-1} at -24 h to 7.2 mmol l^{-1} at 0 h , and 9.5 mmol l^{-1} at 24 h . Lactate concentration decreased temporarily when the drops were supplemented with fresh medium, but the changes were not statistically significant. The mean concentrations ($\pm \text{SEM}$) of glucose and lactate in the bovine oviductal epithelial cell conditioned media were $1.7 \pm 0.4 \text{ mmol l}^{-1}$ and $3.1 \pm 0.6 \text{ mmol l}^{-1}$, respectively. Except at -24 h , and immediately after supplementation with fresh medium, the concentration of glucose in the co-culture droplets was below the lower 95% confidence limit for glucose concentration in the conditioned medium (0.9 mmol l^{-1}). Except at -24 h , the concentration of lactate in the co-culture droplets was above the upper 95% confidence limit for lactate concentration in the conditioned medium (4.1 mmol l^{-1}) during the entire co-culture period.

Discussion

The timing of development of the cattle embryos in this study was consistent with our observations that development to the 16-cell, morula and blastocyst stages is delayed by 24 h in conditioned medium (Grisart *et al.*, 1994) compared with that in oviductal cell co-culture (Rieger *et al.*, 1992a). Moreover, the mean number of cells in blastocysts produced by culture in conditioned medium was significantly lower than in blastocysts produced by co-culture (56.7 ± 3.3 versus 96.8 ± 6.1 ; $P \leq 0.0001$), even though the blastocysts produced by culture in conditioned medium were 24 h older. This finding agrees closely with the observations of Trounson *et al.* (1994), who observed that the mean number of cells is significantly greater in blastocysts produced by bovine oviductal cell co-culture than in those cultured in conditioned medium.

The lower number of cells in the blastocysts produced by culture in conditioned medium suggests that the delay in development between the four-cell and 16-cell stages is associated with a decrease in subsequent developmental potential of the embryo. Yadav *et al.* (1993) showed that cattle embryos that are late in undergoing the first cleavage division

Table 5. The effect of co-culture with bovine oviductal epithelial cells or culture in conditioned medium, and stage of development on the metabolism per cell of glutamine by cattle embryos

Stage	Culture treatment					
	Co-culture		Conditioned medium		Pooled	
	Number of embryos	Metabolism (pmol per cell per 3 h)	Number of embryos	Metabolism (pmol per cell per 3 h)	Number of embryos	Metabolism (pmol per cell per 3 h)
Four-cell	12	0.8 ± 0.1	12	1.1 ± 0.2	24	0.9 ± 0.1
16-cell	7	0.2 ± 0.0	8	0.1 ± 0.0	15	0.1 ± 0.0 ^a
Morula	12	0.2 ± 0.0	11	0.2 ± 0.0	23	0.2 ± 0.0
Blastocyst	11	0.1 ± 0.0	9	0.1 ± 0.0	20	0.1 ± 0.0

Metabolism values are means ± SEM.

^aSignificantly different from the preceding stage in the same column ($P \leq 0.001$).**Table 6.** The effect of co-culture with bovine oviductal epithelial cells or culture in conditioned medium, and stage of development, on the ratio of glucose to glutamine metabolism by cattle embryos

Stage	Culture treatment					
	Co-culture		Conditioned medium		Pooled	
	Number of embryos	Metabolism ratio	Number of embryos	Metabolism ratio	Number of embryos	Metabolism ratio
Four-cell	12	0.3 ± 0.1	12	0.4 ± 0.2	24	0.4 ± 0.1
16-cell	7	0.6 ± 0.0	9	3.3 ± 0.9 ^{ab}	16	2.1 ± 0.6
Morula	12	4.7 ± 0.4 ^a	11	6.2 ± 0.9 ^a	23	5.4 ± 0.5
Blastocyst	11	5.3 ± 0.7	12	6.9 ± 0.7	23	6.1 ± 0.5

Metabolism ratios are means ± SEM.

^aSignificantly different from the preceding mean in the same column ($P \leq 0.0005$).^bSignificantly different from the same stage in the co-culture group ($P \leq 0.0001$).

have a reduced number of cells at the blastocyst stage. Cattle embryos that are late in reaching the blastocyst stage are less likely to hatch *in vitro* or develop after transfer to recipient cows (Massip *et al.*, 1995; J. W. Pollard and S. P. Leibo, personal communication).

The mechanism by which oviductal cells or other somatic cells can improve the development of early mammalian embryos *in vitro* is unknown. Early embryos have receptors for a variety of cellular growth factors (Schultz and Heyner, 1993), and it has been suggested that somatic cells may secrete tissue-specific or non-specific growth factors that can stimulate embryo development (reviewed by Gandolfi, 1994). However, there is no direct evidence for this, and it has therefore been suggested that somatic cells simply remove deleterious substances, notably glucose, from the medium (reviewed by Bavister, 1992; Bavister, 1995). This view is supported by the observations that embryos develop better in simple glucose-free media such as CZB (Chatot *et al.*, 1989), HECM (McKiernan *et al.*, 1991), CR2 (Rosenkrans *et al.*, 1993), and KSOM (Erbach *et al.*, 1994) than in complex glucose-containing media. It is therefore interesting that the oviductal cell

cylinders used in the co-culture reduced the glucose concentration in TCM-199 more than did conditioning on oviductal cell monolayers. The more rapid development and greater number of cells in blastocysts of embryos in co-culture compared with those cultured in conditioned medium is consistent with the observation that the development of sheep embryos is impaired by glucose concentrations greater than 1.5 mmol l⁻¹ (Thompson *et al.*, 1992).

The higher lactate concentration in the co-culture droplets compared with the conditioned medium may also have contributed to the relatively better embryo development in co-culture. Lactate can be used as an energy substrate by mouse and hamster embryos, and may also serve to maintain the proper redox balance and intracellular pH (reviewed by Leese, 1991; Bavister, 1995). Our results are consistent with the observations that lactate concentrations of 3.3–10 mmol l⁻¹ favour the development of sheep (Thompson *et al.*, 1993) and cattle embryos (Takahashi and First, 1992; Rosenkrans *et al.*, 1993).

The results also suggest that the culture conditions can affect the ability of the embryo to metabolize glucose. For the

Fig. 1. The effect of time on the mean concentrations ($\pm$ SEM) of glucose ($\circ$) and lactate ($\bullet$) in bovine oviductal epithelial cell co-culture droplets. Embryos are normally introduced at time 0. The arrows indicate the times when the co-culture droplets were supplemented with fresh medium. *Significantly different from the preceding sample mean for the same substrate ($P \leq 0.05$).

co-cultured embryos, glucose metabolism was low at the four-cell and 16-cell stages and then increased significantly through the morula and blastocyst stages, which is consistent with previous observations (Rieger *et al.*, 1992a, b). However, the pattern of glucose metabolism by the embryos cultured in conditioned medium was significantly different, in that embryos metabolized significantly more glucose overall than did those in co-culture, on both a per embryo, and a per cell, basis. In particular, glucose metabolism increased significantly between the four-cell and 16-cell stages in the embryos cultured in conditioned medium, whereas there was no significant change in the co-cultured embryos. Consequently, the metabolism of glucose at the 16-cell stage was four times greater by embryos cultured in conditioned medium than by co-cultured embryos. There is a large increase in glucose uptake between the eight-cell and 16-cell stages in cattle embryos produced by co-culture, and consequently the proportion of glucose metabolized by those embryos is at a minimum at the 16-cell stage (Rieger *et al.*, 1992b). It is not yet clear whether the embryos cultured in conditioned medium took up more glucose than did the co-cultured embryos, or metabolized a larger proportion of the glucose that they absorbed.

The metabolism of glucose was directly related to the glucose concentration in the culture medium; glucose concentration was higher in the conditioned medium and glucose metabolism was higher in embryos cultured in the conditioned medium. This may suggest that the enzymes involved in glucose transport or metabolism in the early cattle embryo can be induced or activated in response to high glucose or low lactate concentrations in the culture medium. However, whereas the conditioned medium was serum-free, the co-culture medium contained 10% serum, which may have had a more direct and unknown effect on glucose metabolism by the embryos. In addition, it seems likely that the actively metabolizing oviductal cells would significantly reduce the oxygen concentration, which could also affect the glycolytic enzymes. Whatever the mechanism, it is clear that glucose metabolism by

the embryos was affected by the prior culture conditions. To the best of our knowledge, this is a novel observation for embryos of any species.

In contrast to the significant difference in the patterns of glucose metabolism, there were no significant differences in the patterns of glutamine metabolism between the embryos cultured in the two systems. There was, therefore, no apparent relationship between oxidative metabolism and the numbers of cells at the blastocyst stage. Glutamine metabolism is increased by cryopreservation treatments in both horse (Rieger *et al.*, 1991) and cattle (Rieger *et al.*, 1993) blastocysts, possibly a result of disturbances in mitochondrial function. However, the present results provide no evidence to suggest that this is true of the embryos cultured in conditioned medium.

In conclusion, glucose concentrations are reduced and lactate concentrations are increased by both oviductal cell co-culture or conditioning on oviductal cell monolayers. However, the glucose concentration is lower, and the lactate concentration is higher, in oviductal cell co-culture than in medium conditioned by oviductal cells. Development is delayed, and there are fewer cells in the blastocysts in embryos cultured in conditioned medium compared with those of co-cultured embryos. Glucose metabolism is greater in cattle embryos cultured in conditioned medium than in co-cultured embryos, suggesting that the activity of enzymes involved in glucose transport or metabolism in early cattle embryos is affected by the culture conditions. These observations suggest that the higher rate of glucose metabolism induced by culture in conditioned medium is unfavourable for subsequent development. However, further investigation is necessary to determine the specific mechanism(s) responsible for the effect on the glycolytic activity of cattle embryos, and whether a high rate of glucose metabolism leads to delayed development and fewer cells or, alternatively, that the observations have a common cause.

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