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Glucose, glutamine and inorganic phosphate in early development of the pig embryo *in vitro*

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Summary. Pig embryos at the 1- or 2-cell stage (before the 'block' to development *in vitro*) were cultured in 8 different media derived from Krebs'–Ringer–bicarbonate medium. A 2 × 2 × 2 factorial arrangement was used for the treatments, with glucose, glutamine and phosphate being the major effects tested. Embryos were obtained from sows approximately 44–48 h after the observation of oestrus, with the majority being at the 1-cell stage. Embryos from each female were randomly assigned to each treatment. After in-vitro culture, all embryos were scored for the stage of development attained and stained to determine final cell number. Significant effects were evident due to female, glucose, glutamine, a phosphate × glucose interaction and a glutamine × glucose interaction. None of the media components tested was inhibitory to embryo development. The greatest development (45–60% morula or blastocyst) was achieved with glucose and glutamine (both alone and in combination) in the media, demonstrating that an amino acid can serve as the sole energy source for complete preimplantation embryonic development *in vitro*.

Keywords: embryo; pig; in vitro culture; glutamine; glucose; phosphate

Introduction

Maintaining embryo viability during in-vitro culture is a key to the application of such biotechnologies as embryo splitting, nuclear transplantation and gene transfer by DNA microinjection or retroviral infection. Embryos from most species exhibit less than optimal development *in vitro* and may 'block' during development at specific cell stages (Bavister, 1987). Because embryos from these animals develop *in vivo*, the culture medium or environmental conditions used for in-vitro culture must be inferior for supporting normal development.

Pig embryos can develop from the zygote to the blastocyst stage *in vitro*, but the proportion of embryos obtained before the 4-cell stage that develop to the blastocyst is low (Davis, 1985). This block to development can be overcome *in vitro* by co-culturing pig embryos with oviducal epithelial cells (White *et al.*, 1989), culturing embryos in medium supplemented with pig oviducal fluid (Archibong *et al.*, 1989) or placing the embryos in mouse oviducts maintained in organ culture (Krisher *et al.*, 1989a, b). While these methods are simple and repeatable, improvement of the defined culture environment remains critical.

Schini & Bavister (1988) have shown that phosphate and glucose are responsible for the 2-cell block in the hamster. An inhibitory effect for glucose has also been reported for the mouse (Chatot *et al.*, 1989) and the hamster (Seshagiri & Bavister, 1989a, b). These studies have also demonstrated that amino acids can serve as sole energy sources for development of hamster embryos from the

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8-cell to blastocyst stage and that glutamine plays an important role in early development of mouse and hamster embryos *in vitro*. Davis & Day (1978) reported that deletion of glucose from culture medium still permitted development of pig embryos from the 4-cell to the blastocyst stage *in vitro*. In the present study, we have examined the effects of glucose, glutamine and phosphate on the development of pig embryos.

Materials and Methods

Eight media were formulated by modifying Krebs'–Ringer–bicarbonate medium (Davis & Day, 1978) (Table 1). The calculated osmolarity of each medium was adjusted to 305–306 mosmol by adjusting the NaCl concentration (114·73 to 119·20 mM). All medium preparations were tested with an osmometer for uniformity as a quality control measure. Media were stored in sterile glass containers at 4°C and used within 7–10 days of preparation. These media were used in a 2 × 2 × 2 factorial arrangement to test the effects of glucose (5·5 mM or 0 mM), phosphate (KH_2PO_4, 1·19 mM or 0 mM) and glutamine (1 mM or 0 mM) on 1- and 2-cell embryos (before the block).

Table 1. Complete culture media used for the culture of early stage pig embryos

Ingredient	mM	g/l
NaCl	114·73	6·705
KCL	4·78	0·356
$CaCl_2$	1·70	0·189
KH_2PO_4*	1·19	0·162
$MgSO_4 \cdot 7H_2O$	1·19	0·294
$NaHCO_3$	25·07	2·106
Glucose*	5·55	1·000
Glutamine*	1·00	0·146
Bovine serum albumin (Fraction V)		4·000
Penicillin/streptomycin (10 000 Units/ml and 10 mg/ml)—10 ml/l		
Phenol red (1%)—1 ml/l		

*Treatment media were compounded by including or not including these ingredients.

Crossbred production sows (Landrace, Yorkshire and Chester White triple crosses) used in the present study were checked twice daily for oestrus with intact Duroc boars. Sows were mated at the time of detected oestrus (Day 0 of gestation) and 24 h later. Embryos from mated sows were surgically collected from each oviduct according to the method of Vincent *et al.* (1964) 44–48 h after the onset of oestrus. One- or 2-cell embryos were washed three times in culture medium lacking glucose, phosphate and glutamine and assessed for viability by visual inspection with the light microscope: 1-cell ova with visible polar bodies and/or spermatozoa embedded in the zona pellucida and 2-cell embryos with blastomeres of equal size were used.

Each sow was used as a random block, contributing an equal number of viable embryos to each treatment. Sows were not used when fewer than 8 viable embryos were recovered. One sow contributed 16 embryos. Consequently, embryos from 19 females (160 embryos, 1- or 2-cell stages) were cultured in the 8 media, 20 embryos/medium. The embryos were cultured individually in 1 ml medium in Corning Cell Well™ plates (No. 25820, Corning Glass Works, Corning, NY, USA) in a sealed, humidified glass desiccator gassed with 5% O_2–5% CO_2–90% N_2 and maintained at 37°C in a tissue culture incubator. The length of culture *in vitro* depended on the stage of development of the embryos at recovery (1 cell = 6 days; 2 cell = 5 days). At the end of the culture period, embryos were scored for morphological stage of development by light microscopy and for final cell number after staining and counting of nuclei using the technique of Pursel *et al.* (1985).

Each embryo was given a numerical score based on its morphological stage of development as follows: dead or degenerate = 1; cleavage = 2; morula = 3; blastocyst = 4. These scores were used to calculate the morphological stage of development attained for embryos in each treatment. Statistical analyses of the data were accomplished with the General Linear Model of the Statistical Analysis System (SAS, Cary, NC, USA). The complete model included the main effects (female, glucose, glutamine, phosphate), interactions (between glucose, glutamine and phosphate) with initial cell number as a covariate. Comparisons between least square means were made by *t* tests.

Results

Embryo development for each treatment medium is shown in Table 2. Generally, all treatments with glucose or glutamine or both in the medium were similar. One exception was the treatment with phosphate and glucose (lacking glutamine) which is the usual culture medium. In this case, development appeared to be reduced, being significantly different from a number of the other media. Medium lacking any exogenous energy source (i.e. lacking both glucose and glutamine) did not support development much beyond the 4-cell stage, although the medium with phosphate alone did support some degree of development past cleavage (20% morula and blastocyst).

Table 2. Summary of the effects of different media with glucose (±), glutamine (±) and phosphate (±) for culture of 1 and 2-cell pig embryos

Medium				Stage of development (cell no.)					LS mean	
PO	G	Gln	No. of embryos	Deg	Cl	M	B	% M + B	Stage of development*	Final cell no.†
+	+	+	20	1 (2·0)	9 (8·4)	4 (22·5)	6 (62·5)	50	2·69	25·6
−	+	+	20	0	8 (6·6)	3 (23·3)	9 (53·8)	60	2·99	28·8
+	+	−	20	1 (1·0)	10 (7·1)	7 (18·6)	2 (47·5)	45	2·44	13·3
−	+	−	20	0	8 (6·9)	6 (21·3)	6 (56·0)	60	2·84	24·4
+	−	+	20	0	9 (4·0)	5 (20·6)	6 (55·2)	55	2·79	22·0
−	−	+	20	1 (1·0)	9 (4·8)	6 (24·7)	4 (59·3)	50	2·59	19·9
+	−	−	20	3 (1·3)	13 (3·9)	3 (29·7)	1 (47·0)	20	2·04	7·8
−	−	−	20	8 (1·4)	12 (4·9)	0	0	0	1·54	1·8

PO = phosphate; G = glucose; Gln = glutamine; Deg = degenerate; Cl = cleavage; M = morula; B = blastocyst.
*s.e. of all means, 0·16.
†s.e. of all means, 3·64–3·66.

Significant effects in the statistical model, based on stage of development, were female, glucose, glutamine, and interactions of phosphate with glucose and glutamine with glucose (Table 3). The stage of embryonic development at the beginning of the culture period (1- or 2-cell) did not influence subsequent development *in vitro*. The greatest development (45–60% morulae and blastocysts) was achieved with glucose and/or glutamine (both alone or in combination) in the media (Table 2). The effect of glutamine was independent of the presence of phosphate in the media. The interaction noted for glutamine and glucose indicated that media with glutamine and with or without glucose resulted in equal development, but media without glutamine supported development only when glucose was present. Phosphate and glucose interacted with each other and a greater degree of development occurred in the absence of phosphate when glucose was present.

Analysis based on the number of cell divisions achieved *in vitro* indicated that significant effects were female, glucose, glutamine and an interaction of phosphate with glucose (Table 3). The greatest numbers of cells were achieved in media containing glucose and glutamine (both alone or in combination). Average cell numbers attained for morulae across all treatments ranged from 19 to 30 cells (nuclei) per embryo, while for blastocysts the averages ranged from 47 to 63 cells (nuclei) per embryo.

Thus, for both stage of development and final cell number data, glucose or glutamine could serve as the sole exogenous energy source. Glucose and glutamine in combination were not better than either energy source alone.

Discussion

The majority of the in-vitro embryonic development data has been gathered from studies of the mouse embryo. Several early studies were conducted to elucidate the needs of the preimplantation

Table 3. Analysis of variance of the complete data set for 160 pig embryos cultured in 8 different media with dependent variables of stage of development attained or final cell number

		Stage of development		Final cell no.	
Source	d.f.	Mean square	F value	Mean square	F value
Female	18	2·02	4·09***	1518·58	5·79***
Phosphate (PO)	1	0·00	0·00	96·10	0·37
Glucose (G)	1	9·08	18·37***	3687·35	14·05***
Glutamine (Gln)	1	11·07	22·40***	5436·61	20·72***
PO × G	1	4·90	9·91**	1254·40	4·78*
PO × Gln	1	0·10	0·20	38·03	0·14
G × Gln	1	4·30	8·70**	457·13	1·74
PO × G × Gln	1	0·40	0·81	354·03	1·35
Initial cell no.	1	0·85	1·72	346·63	1·32
Error	133	0·49		262·42	

*$P < 0{\cdot}05$; **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$.

mouse embryo, in terms of nitrogen and energy requirements, as well as the physico-chemico requirements (for review see: Biggers *et al.*, 1967; Brinster, 1969, 1973; Biggers, 1987). The mouse embryo undergoes temporal changes in cellular respiratory requirements. At the 1- and 2-cell stages pyruvate and lactate can support development while glucose cannot. Additionally, early stage embryos can utilize a portion of the available glucose via the pentose phosphate pathway for the production of reducing compounds and phosphorylated pentose sugars. The activity of this system declines as development proceeds to the blastocyst stage (O'Fallon & Wright, 1986). During the later stages of development, glucose is utilized preferentially, beginning at the morula and blastocyst stages. One possible reason behind the differential energy substrate requirements may be the lack of a functional glycolytic pathway in the early cleavage stages. A block to glycolysis is suspected due to decreased 6-phosphofructokinase activity. This block is removed with further embryonic development, as indicated by increased enzyme activity (Barbehenn *et al.*, 1974, 1978).

The hamster embryo exhibits several blocks to development before blastocyst formation (Bavister, 1987, 1988). Major deviations in the formulation of culture media for hamster embryos have produced surprising results. The inclusion of phosphate and glucose in hamster embryo culture medium has been found to be strongly inhibitory; removal of these compounds allowed for greater development of 2-cell embryos to the 8-cell/morula stages and greater development of 8-cell embryos to blastocysts (Schini & Bavister, 1988; Seshagiri & Bavister, 1989a, b). A requirement for vitamins (inositol, pantothenate, and choline) to promote hatching of hamster embryos *in vitro* has also been demonstrated (Kane & Bavister, 1988).

The addition of glutamine and the deletion of glucose from media has also resulted in increased in-vitro development of 1-cell mouse embryos that exhibit a block at the 2-cell stage. Glutamine was found to be important for the transition through the developmental block while glucose was inhibitory. The requirement for glutamine was lost at the 4- to 8-cell transition and glucose became necessary for further embryonic development (Chatot *et al.*, 1989).

In the present study, media containing glucose, as energy source, permitted a greater number of pre-block pig embryos to reach the morula and blastocyst stages compared with media lacking an energy source. The intrepretation of the significance of glucose for pig embryo culture *in vitro* is beset by the conflicting results reported by Pope (1972) on in-vitro culture of 1–4 cell pig embryos in Brinster's medium for ovum culture-2 + glucose. The result of the present study regarding the influence of glucose on the trend of embryonic development is similar to that obtained in the first experiment conducted by Pope (1972). However, the percentage of blastocyst formation observed in this study is higher than that observed by Pope (1972). It is possible that media containing glucose may have provided more energy for embryonic development via the glycolytic pathway

after two cleavage divisions, compared with media without glucose. According to Flood & Wiebold (1988) the early pig embryo (1–4-cell stage) uses the pentose phosphate pathway for the metabolism of glucose to a greater extent than glycolysis. However, the utilization of glucose through the glycolytic pathway increases with embryonic development. Because glucose and inorganic phosphate together in culture medium did not cause an increase in percentage of morula and blastocyst formation beyond that observed when glucose alone was present, the inorganic phosphate required for the phosphorylation of glucose for the onset of glycolysis may have been endogenous. The highly significant effect of female (sow) has been noted previously (Krisher *et al.*, 1989a; Archibong *et al.*, 1989) and is either due to genetic differences among sows or effects of surgery on individual sows.

The end product of glycolysis is pyruvate or lactate which have been shown to inhibit pig embryo development (Davis & Day, 1978). In the in-vitro embryo culture system used in this study, in which one embryo was cultured in 1 ml medium, the amount of pyruvate produced by the embryo probably was not up to the threshold required to inhibit embryonic development. In the oviduct, pyruvate produced as a result of glycolytic activity within the embryo may be taken up by the cells of this organ, thus reducing the likelihood of pyruvate inhibiting embryonic development. Bavister (1988) and Krisher *et al.* (1989b) have suggested that oviducal cells play a role in the removal of toxic factors in the environment of the early developing embryo.

Glutamine alone or in combination with glucose or inorganic phosphate sustained the development of pig embryos to the morula and blastocyst stages to the same extent as did glucose. Glutamine has been shown to be an energy substrate for a variety of somatic cells *in vitro* (Eagle *et al.*, 1955; Zielke *et al.*, 1978; Reitzer *et al.*, 1979). In terms of uptake and utilization by mammalian oocytes and embryos, glutamine has been shown to be beneficial for hamster oocytes and embryos, as well as rabbit oocytes *in vitro* (Gwatkin & Haidri, 1973; Bavister *et al.*, 1983; Bae & Foote, 1975; Carney & Bavister, 1987). Mouse embryos can also utilize glutamine as an energy source (Brinster, 1971, 1973; Chatot *et al.*, 1989, 1990).

The ability of glutamine to support embryo development in the absence of glucose demonstrates its ability to replace glucose as the carbon source for energy production. These findings are supported by those of Stone *et al.* (1984) who obtained greater development of 4–8-cell pig embryos *in vitro* in minimal essential medium (MEM) containing glutamine, over that of Krebs'–Ringer bicarbonate medium supplemented with glucose. However, Meyen *et al.* (1989) found no beneficial effect of glutamine on development of morulae and blastocysts. According to these investigators, an inhibitory effect was observed with the inclusion of the amino acid and vitamin complexes present in MEM. These observations, in light of the present findings, suggest that a difference may exist in the sensitivity of the pig embryo to the MEM components over time, specific amino acids or vitamins, and that glutamine appears to exert a positive effect, particularly at the early stages of development. However, Rosenkrans *et al.* (1989) concluded that glutamine improved development of pig blastocysts *in vitro*. All media in the present study contained bovine serum albumin which may contain substrates for energy production as contaminants. However, medium lacking glutamine and glucose did not support much embryo development. The final cell numbers attained by pre-block pig embryos cultured *in vitro* in the present study are similar to those observed by Krisher *et al.* (1989b) with pig zygotes in mouse oviducts in organ culture, and in-vitro culture of pig 4-cell (Davis & Day, 1978; Niemann *et al.*, 1983), and morulae and blastocysts (Meyen *et al.*, 1989) stages.

The presence of glucose in the present study was found to be necessary (in the absence of glutamine) for in-vitro development to proceed, and was not inhibitory to development, as it is for mouse and hamster embryos (see above). The presence of phosphate in the medium had no overt beneficial or negative effects on development, but an interaction between phosphate and glucose was observed. This interaction indicated a negative effect of phosphate in those media in which glucose was present, and this may be analogous to the inhibitory effects seen with hamster embryos (Schini & Bavister, 1988; Seshagiri & Bavister, 1989a, b).

In conclusion, this study demonstrated that a greater proportion of 1- and 2-cell pig embryos developed in the presence of glucose and glutamine (both alone and in combination) than when both were absent, as measured by stage of embryonic development and the number of cell divisions achieved during the culture period. No inhibitory effects were observed for glucose or glutamine. Development of 1- or 2-cell embryos through the 4-cell in-vitro development block to morula and blastocyst stages was demonstrated in treatments in which glutamine was the sole exogenous energy source.

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