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Research

Use of energy substrates by various stage preimplantation pig embryos produced *in vivo* and *in vitro*

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The aim of *in vitro* embryo systems is to produce embryos of comparable quality to those derived *in vivo*. Comparison of embryo metabolism as an indicator of viability may be useful in optimization of culture conditions. The aim of the present study was to determine glucose, glutamine and pyruvate use by various stage pig embryos produced *in vitro* and *in vivo*. The results indicate that pig embryos use glucose via glycolysis in significant amounts at all stages examined, regardless of embryo origin. *In vitro*-derived embryos have significantly increased glycolytic activity after the eight-cell stage, whereas *in vivo*-derived embryos have increased glycolysis at the blastocyst stage. *In vivo*-derived embryos have higher rates of glycolysis compared with *in vitro*-derived embryos. Glucose usage through the Krebs cycle for *in vitro*- and *in vivo*-derived embryos increased significantly at the blastocyst stage. Pig embryos produced *in vitro* used constant amounts of glutamine throughout development, whereas *in vivo*-derived embryos increased glutamine usage after the eight-cell stage. Pyruvate use was minimal at all stages examined for both *in vitro*- and *in vivo*-derived pig embryos, showing significant increases at the blastocyst stage. Krebs cycle metabolism of pyruvate, glutamine and glucose by *in vivo*-derived embryos was higher than that by *in vitro*-derived embryos. Current *in vitro* culture conditions produce pig embryos with altered metabolic activity, which may compromise embryo viability.

Introduction

The recent successes in cloning and transgenic pig production, and the immense potential these present for xenotransplantation have reaffirmed the need for a more efficient *in vitro* embryo production system for pigs. Embryo metabolism is a valuable tool for evaluating the efficiency of these *in vitro* systems. Embryo metabolism may serve as an indicator of embryo viability (Renard *et al*., 1980; Conaghan *et al*., 1993; Lane and Gardner, 1996), as well as provide crucial information to support the formulation of a more appropriate culture medium. However, there are relatively few publications describing metabolism of pig embryos. In addition, the developmental competence of *in vitro*-derived pig embryos is low compared with that of other domestic species.

Understanding the metabolic needs of preimplantation embryos is vital to optimize growth. If the necessary energy substrates are not present in sufficient concentrations or at the appropriate time, the embryo will be unable to develop. One recurring pattern observed in embryo metabolism is increased glycolysis and glucose usage as preimplantation development progresses (Flood and Wiebold, 1988; Thompson *et al*., 1991; Rieger *et al*., 1992a,b). However, species-specific preferences are evident. Glucose is inhibitory at all stages of hamster embryo development (Schini and Bavister, 1988a; Seshagiri and Bavister, 1989). Glucose can also have an inhibitory effect on murine (Chatot *et al*., 1989, 1990) and bovine (Kim *et al*., 1993) embryos when present before the maternal–zygotic transition. Developing murine embryos prefer pyruvate as their main energy source until they reach the blastocyst stage (Leese and Barton, 1984), whereas lactate is essential for development of hamster (McKiernann *et al*., 1991) and bovine (Rosenkrans *et al*., 1993) embryos. Amino acids serve as an additional energy source for developing embryos. Glutamine is important in the transition through the developmental block in hamster embryos (Carney and Bavister, 1987). Mouse embryos increase glutamine usage throughout development (Gardner *et al*., 1989; Chatot *et al*., 1990). There is a significant increase in glutamine metabolism accompanying blastocyst expansion in bovine embryos (Rieger *et al*., 1992b).

Lactate inhibits preimplantation development of pig embryos (Davis and Day, 1978; Davis, 1985) and pyruvate is not necessary for development to the blastocyst stage (Petters *et al*., 1990; Petters and Reed, 1991). Both glucose and glutamine, alone or in combination, can support preimplantation development of pig embryos (Petters *et al*., 1990). Glucose is metabolized equally via aerobic and anaerobic pathways by early cleavage stage pig embryos developed *in vivo*, although glycolysis becomes the dominant pathway as development progresses (Flood and

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Weibold, 1988). From this information, it is evident that pig embryos have changing metabolic needs as they develop and undergo the accompanying morphological and biochemical changes associated with growth, such as the maternal–zygotic transition, compaction and blastocoel development.

There are many differences between embryos produced *in vitro* and *in vivo*. *In vitro*-derived embryos show delayed development, altered cell number and allocation, and altered morphology (for a review, see Thompson, 1997). Usage of substrates by *in vitro*-and *in vivo*-derived embryos also differs in some species (Thompson, 1991; Khurana and Niemann, 2000). *In vitro*-derived embryos tend to have increased glycolytic activity at early cleavage stages and increased lactate production compared with their *in vivo* counterparts, which alters normal redox potential and intracellular pH (Gardner and Leese, 1990). This is probably the result of suboptimal maturation, fertilization or culture conditions. However, all metabolic measurements are conducted in an *in vitro* environment so the physiological metabolism *in vivo* may differ. The aim of the present study was to examine glucose usage through glycolysis and the Krebs cycle, and glutamine and pyruvate usage through the Krebs cycle, of *in vitro*- and *in vivo*-derived pig embryos at the two-cell, eight-cell, morula and blastocyst stages of development.

Materials and Methods

In vitro *maturation*

Pig ovaries were obtained from a local abattoir and transported in 0.9% (w/v) saline at a temperature of approximately 30°C. Oocytes were collected from antral follicles 3–8 mm in diameter using a 10 ml syringe or aspiration pump (10 mm Hg; 28 ml min^{-1}) and an 18-gauge needle. Cumulus–oocyte complexes (COCs) with at least two layers of compact cumulus cells and even cytoplasm were washed twice in synthetic oviductal fluid (SOF) Hepes-buffered medium supplemented with 1.0% (v/v) PSA (100 U penicillin ml^{-1}, 100 μg streptomycin ml^{-1}, 0.25 ng amphotericin ml^{-1}; GibcoBRL, Grand Island, NY). Selected COCs were matured (50 per 500 μl) in four-well plates (Nunc, Roskilde) covered with 400 μl mineral oil (Sigma, St Louis, MO). Maturation medium (Abeydeera *et al*., 1998) was completely defined TCM199 medium (Gibco) supplemented with 0.01 U pig LH ml^{-1} and FSH (Sioux BCHM, Sioux Center, IA), 10 ng epidermal growth factor ml^{-1} (EGF; Sigma), 0.91 mmol pyruvate l^{-1} (Sigma), 0.57 mmol cysteine l^{-1} (Sigma), 3.05 mmol glucose l^{-1} (Sigma), 1.0% (v/v) PSA and 0.01% (w/v) polyvinylalcohol (PVA; Sigma). Oocytes were matured for 45–47 h in 5% CO_2 in air at 39°C. No selection was performed after maturation.

In vitro *fertilization*

Frozen pelleted semen was thawed and washed in 10 ml Dulbecco's phosphate buffered saline (D-PBS; Gibco) without $CaCl_2$, supplemented with 1.0% (v/v) PSA at 39°C and centrifuged at 1000 ***g*** for 4 min. The supernatant was decanted and the spermatozoa were washed two additional times in 10 ml D-PBS. Sperm concentration was determined using a haemocytometer and adjusted to 1.0×10^7 spermatozoa ml^{-1} using modified Tris-buffered medium (mTBM; Abeydeera and Day, 1997) supplemented with 3.5 mmol caffeine l^{-1} (Sigma) and 0.2% (w/v) fraction V BSA (Sigma). The spermatozoa were preincubated for 2.5 h in 5% CO_2 in air at 39°C to facilitate capacitation. Oocytes were denuded after maturation in SOF Hepes-buffered medium with hyaluronidase (0.01 μg ml^{-1}) immediately before fertilization and placed (20 oocytes per 50 μl droplet) in mTBM. Oocytes were fertilized by adding 50 μl of the sperm suspension to the droplets containing oocytes to give a final concentration of 5.0×10^6 spermatozoa ml^{-1}. Oocytes were fertilized for 4 h in 5% CO_2 in air at 39°C. No selection was performed after fertilization.

In vitro *culture*

Presumptive zygotes were washed vigorously three times in culture medium to remove excess spermatozoa and cultured (8–10 per 50 μl droplet) under mineral oil in NCSU23 (Petters and Wells, 1993; 5.55 mmol glucose l^{-1}, 7.0 mmol taurine l^{-1}, 1.0 mmol glutamine l^{-1}) without hypotaurine supplemented with 0.4% (w/v) Pentex BSA (Serologicals, Kanakee, IL) in 5% CO_2, 10% O_2 and 85% N_2 at 39°C. Embryos were divided randomly into four dishes, one for each cell stage to be examined. Embryos were cultured for 20–22 h, 78–80 h, 118–120 h or 144 h to obtain two-cell, eight-cell, morula or blastocyst stage embryos, respectively.

In vivo *embryo recovery*

Prepubertal gilts ($n = 30$) were synchronized with injections of PG600® (Intervet, Millsboro, DE) at day 200 of age. The gilts were injected with 1000 iu hCG. Sows ($n = 6$) were checked daily for signs of natural oestrus. Embryo donors were artificially inseminated twice at 12 h intervals when in standing oestrus. Sows were inseminated 5–7 days after weaning. At the time of embryo flushes, the pigs were anaesthetized with an initial i.m. injection of 6 ml of an anaesthetic combination consisting of Ketaset® (Fort Dodge Animal Health, Fort Dodge, IA; 5.9 mg kg^{-1}), Telazol® (Fort Dodge; 12.9 mg kg^{-1}) and Rompun® (Bayer, Shawnee Mission, KS; 0.06 mg kg^{-1}), followed by a 4 ml i.v. injection via the ear vein. Small endotracheal tubes (7.0–7.5 mm inner diameter) were inserted into the nose to be connected to an anaesthesia apparatus, supplying oxygen (2 l min^{-1}) and 5% halothane. A ventral mid-line approach was performed and embryos were flushed from the oviduct or uterus, depending on the cell stage, with Beltsville embryo culture media (BECM-3; Dobrinsky *et al*., 1996) at 39°C. A three-layer closure of the incision was performed on embryo donors. Embryos were flushed at day 2, 4, 5 or 6

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after oestrus to obtain two-cell, eight-cell, morula or blastocyst stage embryos, respectively. Surgical embryo recovery procedures were approved by the University of Illinois and Purdue University Animal Care and Use Committees.

Metabolic measurements

Embryos were washed and placed in a 50 μl droplet of modified SOF-based metabolism medium containing 6.0 mmol glucose l^{-1}, 0.1 mmol taurine l^{-1}, 1.0 mmol glutamine l^{-1}, 0.065 mmol pyruvate l^{-1} and 0.01% (w/v) PVA (Sigma) covered in mineral oil (Sigma). Metabolism medium was formulated to resemble energy substrate concentrations in NCSU23 culture medium. Embryo metabolism was measured using a modification of the hanging-drop technique, described and validated by O'Fallon and Wright (1986) and Rieger *et al.* (1992a,b). The labelled substrates used included pyruvic acid, Na salt [2-C^{14}] (0.257 mmol l^{-1}, specific activity 0.0045 μCi μl^{-1}; American Radiolabeled Chemicals, St Louis, MO), L-[2,3,4-^{3}H] glutamine (0.005 mmol l^{-1}, specific activity 0.25 μCi μl^{-1}; ARC) and D-[6-^{14}C] glucose (0.5 mmol l^{-1}, specific activity 0.028 μCi μl^{-1}; ARC) to measure Krebs cycle activity and D-[5-^{3}H] glucose to measure glycolysis (0.016 mmol l^{-1}, specific activity 0.25 μCi μl^{-1}; Amersham Life Science, Arlington Heights, IL). Substrate concentrations were consistent across experiments for each combination of radiolabels and for both *in vivo*- and *in vitro*-derived embryos. Labelled substrates were dried under nitrogen gas, reconstituted in the metabolism medium described above and allowed to equilibrate at 38.5°C in 5% CO_2 in air. Both *in vivo*- and *in vitro*-derived embryos were washed and held briefly in metabolism medium before measurement of metabolism. Embryos were taken up individually in 2 μl metabolism medium, combined with 2 μl of medium containing the labelled substrates and placed in the cap of a 1.5 ml microcentrifuge tube (Fisher, Pittsburgh, PA). The cap was placed on top of the tube containing 1.5 ml of 25 mmol warmed sodium bicarbonate solution l^{-1}, gassed with 5% CO_2, 10% O_2 and 85% N_2, and incubated for 3 h at 38.5°C. Sham and total count tubes were prepared for each replicate in an identical manner, but containing no embryo. Total count tubes were shaken before incubation.

After the 3 h incubation, the caps were removed and 1.0 ml of the sodium bicarbonate was placed into a scintillation vial containing 200 μl of 0.1 mmol NaOH l^{-1} and refrigerated at 4°C for 16–20 h. Blastocyst and morula stage embryos were recovered for determination of the number of cells. Scintillation fluid (10 ml per vial; Ecolite, ICN, Costa Mesa, CA) was added and vials were counted for 4 min with a scintillation counter programmed for dual-label counting. The amount of substrate metabolized by each embryo was calculated as described by Tiffin *et al.* (1991), accounting for concentrations of labelled and unlabelled substrates.

Cell counts

Morula and blastocyst stage embryos were stained with 0.1 mg Hoechst 33342 DNA stain ml^{-1} (Pursel *et al.*, 1985) for 20 min and cells were counted at × 200 magnification with an inverted Nikon microscope using UV light.

Statistical analysis

The data were analysed using GLM ANOVA. If variances were not homogeneous, as determined by the modified Levene equal variance test, the data were log-transformed before analysis. Differences were determined using a Bonferroni multiple comparison test for normally distributed data, or a Kruskal–Wallis multiple comparison test for non-normally distributed data. The level of significance was $P < 0.05$. Data are mean ± SEM.

Results

In vitro-derived embryos had reduced metabolic activity at all of the cell stages examined compared with *in vivo*-derived embryos when assayed under the conditions described. Both *in vitro*- and *in vivo*-derived pig embryos had increased glycolytic activity throughout preimplantation development (Table 1). Glucose metabolism via glycolysis was used preferentially compared with other substrates and pathways at all cell stages examined in *in vivo*-derived embryos, whereas this was true only after the two-cell stage in *in vitro*-derived embryos. On a per cell basis, glycolytic activity decreased as development progressed in both *in vitro*- and *in vivo*-derived embryos (Table 1). However, glycolytic activity was significantly higher at all stages in *in vivo*-derived pig embryos compared with *in vitro*-derived embryos (Fig. 1a). Glucose metabolism of *in vitro*-derived embryos through glycolysis occurred in significant amounts at all cell stages examined and showed significant increases at both the morula and blastocyst stages (Table 1), whereas glucose metabolism of *in vivo*-derived embryos increased significantly at the blastocyst stage only (Table 1).

The metabolism of glucose via the Krebs cycle in *in vivo*-derived embryos increased significantly at the morula stage and remained high in blastocysts (Table 2). At the two-cell stage, metabolism of glucose via the Krebs cycle was similar between *in vitro*- and *in vivo*-derived embryos (Fig. 1b). However, as development progressed, *in vivo*-derived embryos used more glucose oxidatively than did *in vitro*-derived embryos (Fig. 1b). Glucose was metabolized equally in the Krebs cycle and glycolysis by *in vitro*-derived pig embryos at the two-cell stage. However, glycolysis became the preferred pathway as development progressed. In *in vivo*-derived embryos, glycolysis was the preferred pathway at every cell stage examined. Metabolism of glucose via the Krebs cycle by *in vitro*-derived embryos remained constant throughout development (Table 2) and increased at the morula stage in *in vivo*-derived embryos (Table 2). Glucose use per cell via the Krebs cycle

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Table 1. Glucose metabolism via glycolysis and pyruvate metabolism via the Krebs cycle during preimplantation development of *in vitro*- and *in vivo*-derived pig embryos

Stage of development	*n*	Glycolysis ([5-^{3}H] glucose) pmol embryo^{-1} (3 h)$^{-1}$	Glycolysis ([5-^{3}H] glucose) pmol cell^{-1} (3 h)$^{-1}$	Krebs cycle ([2-^{14}C] pyruvate) pmol embryo^{-1} (3 h)$^{-1}$	Krebs cycle ([2-^{14}C] pyruvate) pmol cell^{-1} (3 h)$^{-1}$	Number of cells ± SEM
In vitro-derived						
Two-cell	12	2.24 ± 0.46^a	1.12 ± 0.23^a	0.30 ± 0.07^a	0.15 ± 0.03^a	2
Eight-cell	21	3.82 ± 0.43^a	0.48 ± 0.05^b	0.38 ± 0.07^a	0.05 ± 0.01^b	8
Morula	32	7.19 ± 0.82^b	0.29 ± 0.03^c	0.47 ± 0.07^{ab}	0.02 ± 0.002^c	25.25 ± 2.86
Blastocyst	41	12.47 ± 1.05^c	0.30 ± 0.03^c	0.57 ± 0.05^b	0.01 ± 0.001^c	47.24 ± 0.68
In vivo-derived						
Two-cell	4	16.27 ± 6.19^{ab}	8.13 ± 3.10^a	0.49 ± 0.17^a	0.25 ± 0.086^a	2
Eight-cell	14	18.20 ± 1.76^a	2.28 ± 0.22^a	1.02 ± 0.13^a	0.13 ± 0.016^a	8
Morula	24	19.40 ± 1.59^a	0.77 ± 0.07^b	1.11 ± 0.16^a	0.04 ± 0.006^b	27.44 ± 1.34
Blastocyst	30	53.02 ± 7.87^b	0.94 ± 0.14^b	1.96 ± 0.22^b	0.03 ± 0.004^b	63.53 ± 5.43

$^{a–c}$Different superscripts indicate significant differences within a column and within a group (*in vitro*- or *in vivo*-derived embryos) ($P < 0.05$).

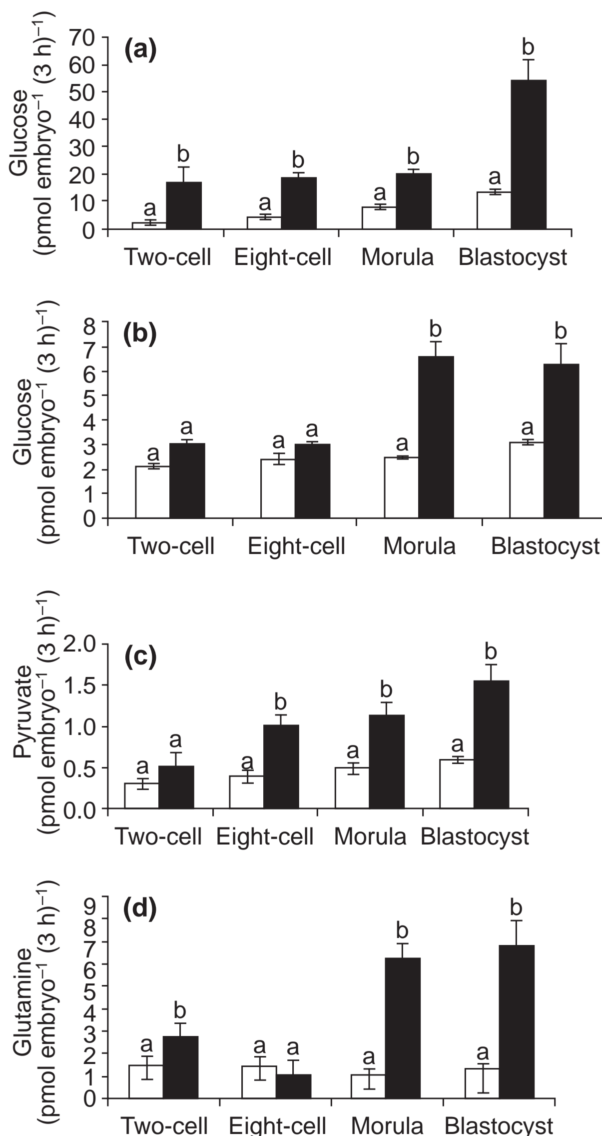

Fig. 1. Differences in use of (a) glucose in glycolysis, (b) glucose in the Krebs cycle, (c) pyruvate and (d) glutamine during preimplantation development in pig embryos derived *in vitro* (□) and *in vivo* (■). Values are mean ± SEM. ^{ab}Different superscripts indicate significant differences between embryo sources within a cell stage ($P < 0.05$).

decreased throughout development in both *in vivo*- and *in vitro*-derived embryos (Table 2).

In vivo-derived pig embryos used minimal amounts of pyruvate at all cell stages examined (Table 1). Significant increases per embryo were observed at the blastocyst stage only (Table 1). Pyruvate metabolism by *in vivo*-derived embryos decreased on a per cell basis at the morula stage (Table 1). After the two-cell stage, pyruvate use by *in vivo*-derived pig embryos was higher than the use by *in vitro*-derived pig embryos (Fig. 1c). Pyruvate use by *in vitro*-derived pig embryos was also minimal throughout preimplantation development (Table 1). As in *in vivo*-derived embryos, a significant increase in pyruvate metabolism occurred in *in vitro*-derived embryos at the blastocyst stage (Table 1). Use of pyruvate by *in vitro*-derived embryos on a per cell basis decreased throughout development (Table 1).

In vivo-derived pig embryos significantly increased glutamine metabolism through the Krebs cycle at the morula stage (Table 2). *In vivo*-derived pig embryos used greater amounts of glutamine than did *in vitro*-derived embryos at all stages except for the eight-cell stage (Fig. 1d). Glutamine metabolism by *in vitro*-derived embryos showed no significant changes throughout development on a per embryo basis, but decreased throughout development when evaluated on a per cell basis (Table 2).

The numbers of cells were similar for both *in vivo*- and *in vitro*-derived morula stage pig embryos. However, *in vivo*-derived blastocysts had significantly higher numbers of cells than did *in vitro*-derived blastocysts. Thus, comparisons of

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Table 2. Glucose and glutamine metabolism via the Krebs cycle during preimplantation development of *in vitro*- and *in vivo*-derived pig embryos

		Krebs cycle ([6-^{14}C] glucose)		Krebs cycle ([2,3,4 ^{3}H] glutamine)		
Stage of development	*n*	pmol embryo^{-1} (3 h)$^{-1}$	pmol cell^{-1} (3 h)$^{-1}$	pmol embryo^{-1} (3 h)$^{-1}$	pmol cell^{-1} (3 h)$^{-1}$	Number of cells ± SEM
In vitro-derived						
Two-cell	9	2.08 ± 0.43^{a}	1.04 ± 0.22^{a}	1.48 ± 0.09^{a}	0.74 ± 0.05^{a}	2
Eight-cell	16	2.37 ± 0.41^{a}	0.30 ± 0.05^{ab}	1.39 ± 0.23^{a}	0.17 ± 0.03^{b}	8
Morula	16	2.41 ± 0.21^{a}	0.09 ± 0.01^{b}	1.01 ± 0.08^{a}	0.04 ± 0.004^{c}	26.2 ± 0.92
Blastocyst	20	2.96 ± 0.28^{a}	0.06 ± 0.01^{c}	1.29 ± 0.12^{a}	0.03 ± 0.002^{c}	56.1 ± 4.48
In vivo-derived						
Two-cell	9	3.01 ± 0.66^{ab}	1.50 ± 0.33^{a}	2.66 ± 0.16^{ab}	1.33 ± 0.08^{a}	2
Eight-cell	17	2.91 ± 0.65^{a}	0.36 ± 0.08^{b}	1.01 ± 0.16^{b}	0.13 ± 0.02^{b}	8
Morula	48	6.47 ± 0.67^{b}	0.25 ± 0.03^{b}	6.17 ± 0.63^{ac}	0.23 ± 0.03^{b}	27.29 ± 1.07
Blastocyst	25	6.19 ± 1.12^{b}	0.10 ± 0.02^{c}	6.74 ± 0.64^{c}	0.11 ± 0.01^{b}	67.92 ± 3.88

[a–c]Different superscripts indicate significant differences within a column and within a group (*in vitro*- or *in vivo*-derived embryos) ($P < 0.05$).

metabolic parameters at this stage are most meaningful when compared on a per cell basis.

Discussion

Comparison of the metabolic activity of pig embryos derived *in vitro* and *in vivo* provides useful information to assess the efficiency of *in vitro* systems for production of pig embryos. The results of the present study indicate that developing *in vitro*-derived pig embryos display metabolism that is significantly different from that of *in vivo*-derived embryos when measured under *in vitro* assay conditions. *In vivo*-derived pig embryos use greater amounts of energy substrates than do *in vitro*-derived embryos in the pathways examined, particularly at the morula and blastocyst stages. Metabolism of endogenous energy stores, such as glycogen and lipid, cannot be assessed using these techniques and may also play a part in energy production in pig embryos.

Under the assay conditions described in the present study, *in vivo*-derived pig embryos used significantly more glucose via glycolysis than did *in vitro*-derived pig embryos at all the cell stages examined. Regardless of embryo source, glycolysis is the predominant metabolic pathway used by developing preimplantation pig embryos. However, more energy is derived for cellular processes by metabolism of glucose via the Krebs cycle. These findings indicate that glycolytic metabolism of glucose may be important to embryos for reasons other than energy production. Metabolism through the pentose phosphate pathway (PPP) is regulated by the flux of glucose through glycolysis. The pentose phosphate pathway, although it does not generate ATP, has several critical functions in the cell. It is responsible for metabolizing glucose, generating ribose 5-phosphate and NADPH. Ribose 5-phosphate is converted to phosphoribosylpyrophosphate (PRPP), a compound needed for the synthesis of purines and for the salvage pathways. In addition, one of the mechanisms that protects cells against harmful oxidants is the glutathione peroxidase reaction. Glutathione peroxidase catalyses the reduction of hydrogen peroxide via reduced glutathione to produce water. NADPH then reduces the resulting oxidized glutathione in a reaction catalysed by glutathione reductase. The pentose phosphate pathway is responsible for using this $NADP^{+}$ to generate more NADPH so that glutathione can be maintained in the reduced state. Pig embryos increase activity of the PPP starting at the morula stage, although as a percentage of total glucose use, PPP activity decreased throughout development (Flood and Wiebold, 1988).

In other species such as mice (Biggers *et al.*, 1967; Leese *et al.*, 1984), cows (Rieger *et al.*, 1992a,b; Kim *et al.*, 1993), sheep (Gardner *et al.*, 1993) and humans (Hardy *et al.*, 1989; Conaghan *et al.*, 1993), lactate and pyruvate are the preferred energy substrates at early cleavage stages. Glucose use in these species is limited until after compaction and glucose has been found to be inhibitory when present before this time (Schini and Bavister, 1988b; Thompson *et al.*, 1992; Kim *et al.*, 1993). However, the results of the present study show that pig embryos metabolize glucose throughout preimplantation development and that *in vivo*-derived pig embryos metabolize more glucose than do *in vitro*-derived embryos. Our findings support a previous report of glucose metabolism by pig embryos *in vivo* (Flood and Weibold, 1988). These findings indicate that glucose is important throughout development of pig embryos. Previous studies indicating an inhibitory effect of glucose on pig embryonic development (Youngs and McGinnis, 1990; Misener *et al.*, 1991) were performed with a combination of glucose and lactate, which may account for the inhibitory effect observed, as it was previously reported that lactate was inhibitory to development of pig embryos (Davis and Day, 1978; Davis,

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1985). The amounts of glucose used via glycolysis by both *in vitro*- and *in vivo*-derived pig embryos in the present study were lower than those reported by Flood and Weibold (1988). This discrepancy may be the result of differences in metabolic measurement technique, including differences in substrate concentration in metabolic measurement medium, which is known to influence metabolism (for a review, see Barnett and Bavister, 1996a,b). Regardless, it is evident that the metabolism of pig embryos is markedly different from that of other species, as pig embryos use glucose as the primary energy substrate throughout preimplantation development.

Flood and Weibold (1988) suggested that the increase in glycolytic activity of pig embryos could be the result of inadequate amounts of glucose uptake to allow sufficient phosphorylation of glucose to glucose-6-phosphate at early cleavage stages. This is probably not the case because comparison of our results with those of a previous study (Gandhi *et al.*, 2001), in which substrate uptake of pig embryos was examined, indicates that pig embryos use only a fraction of the glucose they take up. Flood and Weibold (1988) suggested that there might be inadequate hexokinase activity in early cleavage stage embryos to support increased glycolytic rates. In other species, it appears that hexokinase activity is sufficient to support glycolysis throughout development (Chi *et al.*, 1988; Martin *et al.*, 1993). Instead, glycolysis probably increases as development progresses, as the result of the release of inhibition of phosphofructokinase (PFK) activity. In mouse embryos, ATP concentrations decrease throughout development as the energy demands of the embryo increase (Quinn and Wales, 1971). ATP inhibits phosphofructokinase and, thus, decreasing ATP concentration results in increased phosphofructokinase activity.

The difference in glucose use through glycolysis and the Krebs cycle by both *in vitro*- and *in vivo*-derived embryos observed under *in vitro* assay conditions in the present study indicates that some of the pyruvate derived by glycolysis may be converted to lactate by the embryo. Gandhi *et al.* (2001) reported increasing lactate production by *in vitro*-derived pig embryos as development progressed. Bovine (Khurana and Niemann, 2000) and murine (Lane and Gardner, 1996) embryos produced *in vitro* have increased lactate production compared with *in vivo*-derived embryos. Production of lactate by the embryo may be used to regulate intracellular pH or to adjust the NAD:NADH ratio. Lactate may also have a physiological role in the *in vivo* environment, as lactate concentrations in pig oviductal fluid are higher than the concentrations of pyruvate or glucose (Nichol *et al.*, 1992). *In vivo*-derived pig embryos have been cultured successfully in the presence of lactate (Youngs and McGinnis, 1990; Misener *et al.*, 1991; Dobrinsky *et al.*, 1996) and lactate increased development of IVM–IVF pig embryos (Iwasaki *et al.*, 1999). Thus, lactate, both derived by the embryo and present in the milieu, may be important for development of pig embryos.

Pyruvate is not required for development of pig embryos (Petters *et al.*, 1990; Petters and Reed, 1991). The results of the present study support this conclusion, as metabolism of pyruvate remained minimal throughout all stages of preimplantation development. The concentration of pyruvate in the metabolism medium may influence the metabolism of pyruvate, which may explain why *in vitro*-derived embryos metabolized less pyruvate than did *in vivo*-derived embryos, as there is no pyruvate in NCSU23 culture medium. Pyruvate was included in the medium used for measurement of metabolism to assess the capability of embryos from both sources to metabolize this substrate. In contrast, Gandhi *et al.* (2001) reported significant uptake of pyruvate at the morula and blastocyst stages. It is possible that, *in vitro*, pyruvate is being taken up from the culture medium by the embryo and converted to lactate, instead of being metabolized through the Krebs cycle. Pyruvate use was higher in *in vivo*-derived embryos, indicating that some pyruvate is being metabolized. It is possible that the necessary requirement for pyruvate is met by the metabolism of glucose. However, pyruvate is an antioxidant and may offer the embryo protection against the stress of the environment *in vitro* (O'Fallon and Wright, 1995).

There are conflicting reports about the effects of certain combinations of amino acids on development of pig embryos (Stone *et al.*, 1984; Meyen *et al.*, 1989; Rosenkrans *et al.*, 1989; Kim *et al.*, 1993). Glutamine is beneficial for pig embryonic development and can support development to the blastocyst stage, alone or in combination with glucose (Petters *et al.*, 1990). In the present study, glutamine use through the Krebs cycle by *in vitro*-derived pig embryos remained constant throughout development. However, glutamine use by *in vivo*-derived pig embryos increased as development proceeded. This observation indicates that there may be a deficiency in the concentrations of glutamine in the culture medium or in the enzymes necessary for glutamine uptake or metabolism by *in vitro*-derived pig embryos. Gardner and Lane (1993) suggested that amino acids act as osmoregulators, osmoprotectants, pH regulators and energy sources for preimplantation embryos. The addition of specific amino acids to culture medium enhances development of pig embryos (Petters and Reed, 1991). However, pig embryos can develop to the blastocyst stage in the absence of amino acids (Petters and Wells, 1993). Although amino acids are not required, it appears that glutamine is beneficial and serves some role in normal pig embryonic development.

This is the first study in which the metabolic activity of *in vitro*- and *in vivo*-derived pig embryos has been examined throughout preimplantation development. Pig embryos derived *in vitro* with current culture techniques have significantly altered metabolic activity compared with *in vivo*-derived embryos. Altered metabolic activity may be indicative of suboptimal *in vitro* maturation, fertilization or culture conditions. Using the available metabolic data to formulate a more appropriate culture medium specifically for pig embryos will be of benefit for emerging biotechnologies.

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References

Abeydeera LR and Day BN (1997) Fertilization and subsequent development *in vitro* of pig oocytes inseminated in a modified Tris-buffered medium with frozen–thawed ejaculated spermatozoa *Biology of Reproduction* **57** 729–734

Abeydeera LR, Wang WH, Prather RS and Day BN (1998) Maturation *in vitro* of pig oocytes in protein-free culture media: fertilization and subsequent embryo development *in vitro*. *Biology of Reproduction* **58** 1316–1320

Barnett DK and Bavister BD (1996a) Inhibitory effect of glucose and phosphate on the second cleavage division of hamster embryos: is it linked to metabolism? *Human Reproduction Update* **1** 91–148

Barnett DK and Bavister BD (1996b) What is the relationship between the metabolism of preimplantation embryos and their developmental competence *Molecular Reproduction and Development* **43** 105–133

Biggers JD, Whittingham DG and Donahue RP (1967) The pattern of energy metabolism in the mouse oocyte and zygote *Proceedings National Academy of Sciences* USA **58** 560–567

Carney EW and Bavister BD (1987) Stimulatory and inhibitory effects of amino acids on the development of hamster eight cell embryos *in vitro*. *Journal of In Vitro Fertilization and Embryo Transfer* **4** 162–167

Chatot CL, Ziomek CA, Bavister BD, Lewis JL and Torres I (1989) An improved culture medium supports development of random bred 1-cell mouse embryos *in vitro*. *Journal of Reproduction and Fertility* **86** 679–688

Chatot CL, Tasca RJ and Ziomek CA (1990) Glutamine uptake and utilization by preimplantation mouse embryos in CZB medium *Journal of Reproduction and Fertility* **89** 335–346

Chi MMY, Manchester JY, Yang VC, Curato AD, Stirckler RC and Lowry OH (1988) Contrast in levels of metabolic enzymes in human and mouse ova *Biology of Reproduction* **39** 295–301

Conaghan J, Hardy K, Handyside AH, Winston RML and Leese HJ (1993) Effects of pyruvate and glucose on the development of human preimplantation embryos *in vitro*. *Journal of Reproduction and Fertility* **99** 87–95

Davis DL (1985) Culture and storage of pig embryos *Journal of Reproduction and Fertility Supplement* **33** 115–124

Davis DL and Day BN (1978) Cleavage and blastocyst formation by pig eggs *in vitro*. *Journal of Animal Science* **46** 1043–1053

Dobrinsky JR, Johnson LA and Rath D (1996) Development of a culture medium (BECM-3) for porcine embryos: effects of bovine serum albumin and fetal bovine serum on embryo development *Biology of Reproduction* **55** 1069–1074

Flood MR and Weibold JL (1988) Glucose metabolism by preimplantation pig embryos *Journal of Reproduction and Fertility* **84** 7–12

Gandhi AP, Lane M, Gardner DK and Krisher RL (2001) Substrate utilization in porcine embryos cultured in NCSU23 and G1.2/G2.2 sequential culture media *Molecular Reproduction and Development* **58** 269–275

Gardner DK and Lane M (1993) Amino acids and ammonium regulate mouse embryo development in culture *Biology of Reproduction* **48** 377–385

Gardner DK and Leese HJ (1990) Concentrations of nutrients in mouse oviduct fluid and their effects on embryo development and metabolism *in vitro*. *Journal of Reproduction and Fertility* **88** 361–368

Gardner DK, Clarke RN, Lechene CP and Biggers JD (1989) Development of a noninvasive ultra microfluorometric method for measuring net uptake of glutamine by single preimplantation mouse embryos *Gamete Research* **24** 427–438

Gardner DK, Lane M and Batt P (1993) Uptake and metabolism of pyruvate and glucose by individual sheep preattachment embryos developed *in vivo*. *Molecular Reproduction and Development* **36** 361–368

Hardy K, Hooper MA, Handyside AH, Tutherford AJ, Winston RM and Leese HJ (1989) Non invasive measurement of glucose and pyruvate uptake by individual human oocytes and preimplantation embryos *Human Reproduction* **4** 188–191

Iwasaki T, Kimura E and Totsukawa K (1999) Studies on a chemically defined medium for *in vitro* culture of *in vitro* matured and fertilized porcine oocytes *Theriogenology* **51** 709–720

Khurana NK and Niemann H (2000) Energy metabolism in preimplantation bovine embryos derived *in vitro* or *in vivo*. *Biology of Reproduction* **62** 847–856

Kim JH, Niwa K, Lim JM and Okuda K (1993) Glucose requirements at different developmental stages of *in vitro* fertilized bovine embryos cultured in semi defined medium *Theriogenology* **39** 875–886

Lane M and Gardner DK (1996) Selection of viable mouse blastocysts prior to transfer using metabolic criterion *Human Reproduction* **11** 1975–1978

Leese HJ and Barton AM (1984) Pyruvate and glucose uptake by mouse ova and preimplantation embryos *Journal of Reproduction and Fertility* **72** 9–13

McKiernan SH, Bavister BD and Tasca RJ (1991) Energy substrate requirements for *in vitro* development of hamster 1 and 2 cell embryos to the blastocyst stage *Human Reproduction* **6** 64–75

Martin KL, Hardy K, Winston RML and Leese HJ (1993) Activity of enzymes of energy metabolism in single human preimplantation embryos *Journal of Reproduction and Fertility* **99** 259–266

Meyen BA, Rosenkrans CF and Davis DL (1989) Development of pig blastocysts *in vitro* is altered by serum bovine albumin and amino acids and vitamins *Theriogenology* **31** 463–471

Misener M, Pollard JW and Metzger K (1991) *In vitro* culture of porcine embryos in CZB medium *Theriogenology* **35** 244 (Abstract)

Nichol R, Hunter HF, Gardner DK, Leese HJ and Cooke GM (1992) Concentrations of energy substrates in oviductal fluid and blood plasma of pigs during the peri-ovulatory period *Journal of Reproduction and Fertility* **96** 669–707

O'Fallon JV and Wright RW, Jr (1986) Quantitative determination of the pentose phosphate pathway in preimplantation mouse embryos *Biology of Reproduction* **34** 58–64

O'Fallon JV and Wright RW, Jr (1995) Pyruvate revisited: a non metabolic role for pyruvate in preimplantation embryo development *Theriogenology* **43** 288 (Abstract)

Petters RM and Reed ML (1991) Addition of taurine or hypotaurine to culture medium improves development of one and two cell pig embryos *in vitro*. *Theriogenology* **35** 253 (Abstract)

Petters RM and Wells KD (1993) Culture of pig embryos *Journal of Reproduction and Fertility* **48** 61–73

Petters RM, Johnson BH, Reed ML and Archibong AE (1990) Glucose, glutamine and inorganic phosphate in early development of the pig embryo *in vitro*. *Journal of Reproduction and Fertility* **89** 269–275

Pursel VG, Wall RJ and Rexroad CE (1985) A rapid whole mount staining procedure for the nuclei of mammalian embryos *Theriogenology* **24** 682–691

Quinn P and Wales RG (1971) Adenosine triphosphate content of preimplantation mouse embryos *Journal of Reproduction and Fertility* **25** 133–135

Renard JP, Philippin A and Menezo Y (1980) *In vitro* glucose uptake of glucose by bovine blastocysts *Journal of Reproduction and Fertility* **58** 161–164

Rieger D, Loskutoff NM and Betteridge KJ (1992a) Developmentally related changes in the metabolism of glucose and glutamine by cattle embryos produced and co-cultured *in vitro*. *Journal of Reproduction and Fertility* **95** 585–595

Rieger D, Loskutoff NM and Betteridge KJ (1992b) Developmentally related changes in the uptake and metabolism of glucose, glutamine and

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pyruvate by cattle embryos produced *in vitro*. *Reproduction, Fertility and Development* **4** 547–557

Rosenkrans CF, Zeng GQ, McNamara GT, Schoff PK and First NL (1993) Development of bovine embryos *in vitro* as affected by energy substrates *Biology of Reproduction* **49** 459–462

Schini SA and Bavister BD (1988a) Two-cell block to development of cultured hamster embryos is caused by phosphate and glucose *Biology of Reproduction* **39** 1183–1192

Schini SA and Bavister BD (1988b) Development of golden hamster embryos through the two cell block in chemically defined medium *Journal of Experimental Zoology* **245** 111–115

Seshagiri PB and Bavister BD (1989) Glucose inhibits development of hamster 8-cell embryos *in vitro*. *Biology of Reproduction* **40** 599–606

Stone BA, Quinn P and Seamark RF (1984) Energy and protein sources for development of pig embryos cultured beyond hatching *in vitro*. *Animal Reproduction Science* **7** 405–412

Thompson JG (1997) Comparison between *in vivo* derived and *in vitro* produced pre-elongation embryos from domestic ruminants *Reproduction, Fertility and Development* **9** 341–354

Thompson JG, Simpson AC, Pugh PA, Pugh RW, Jr and Tervit HR (1991) Glucose utilization in sheep embryos derived *in vivo* and *in vitro*. *Reproduction, Fertility and Development* **3** 571–576

Thompson JG, Simpson AC, Pugh PA and Tervit HR (1992) Requirement for glucose during *in vitro* culture of sheep preimplantation embryos *Molecular Reproduction and Development* **31** 253–257

Tiffin G, Rieger D, Betteridge KJ, Yadav BR and King WA (1991) Glucose and glutamine metabolism in pre-attachment cattle embryos in relation to sex and stage of development *Journal of Reproduction and Fertility* **93** 124–132

Youngs CR and McGinnis LK (1990) *In vitro* culture of porcine embryos in Whitten's medium containing varying levels of glucose and bovine serum albumin *Biology of Reproduction* **42** 58 (Abstract)

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