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Effect of exogenous carbohydrates in a serum-free culture medium on the development of *in vitro* matured and fertilized porcine embryos

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Summary

This study was conducted to examine the effect of energy substrates in a serum-free culture medium on *in vitro* development of porcine embryos. Presumptive zygotes derived from *in vitro* fertilization were cultured in glucose-free North Carolina State University (NCSU)-23 medium with glucose, pyruvate, fructose and lactate added to the culture medium singly or in various combinations. In experiment 1, a higher percentage of embryos cleaved (53–63% vs 10–13%) and developed to the blastocyst stage (18–27% vs 0) after the single addition of glucose (5.6 mM), pyruvate (0.5 mM) or lactate (10 mM) than with no energy substrate addition or the addition only of fructose (5.6 mM). In experiment 2, the addition of pyruvate and lactate resulted in higher blastocyst formation (25%) than other combinations (6–22%), while the addition of glucose and pyruvate significantly inhibited blastocyst formation. Increasing lactate concentration, as a single energy supplement, from 5 to 20 mM significantly improved blastocyst formation (7% vs 14–18%), while no benefit was achieved from increasing pyruvate concentration up to 2 mM (experiment 3). Glucose-free NCSU-23 medium supplemented with 0.5 mM pyruvate and 5 mM lactate significantly improved blastocyst formation (28% vs 17%) compared with NCSU-23 medium supplemented with 5.6 mM glucose (experiment 4). In conclusion, pyruvate and lactate are preferable energy substrates to support *in vitro* development of porcine embryos cultured in a serum-free NCSU-23 medium.

Keywords: Energy substrate, Lactate, Pig embryo, Preimplantation development, Pyruvate

Introduction

Effective *in vitro* culture systems for pig embryos are necessary to develop medical biotechnologies such as xenotransplantation. Media composition (Abeydeera *et al*., 1998; Coy *et al*., 1999) and gas atmosphere during culture (Machaty *et al*., 1998; Lee *et al*., 2001; Yoshioka *et al*., 2002) affect the efficacy of such systems. In a previous experiment (Lee *et al*., 2001), a standard protocol was developed which employed a three-gas mixture as a general atmosphere. Developmental capacity of pig embryos was still limited, however, and further refinement of the system was deemed necessary.

Culture media must contain appropriate energy substrates to effectively support embryo development. In our culture system, serum-free North Carolina State University medium (NCSU-23) has utilized glucose as the sole energy source (Swain *et al*., 2002; Yoshioka *et al*., 2002). It has been shown that glucose may either stimulate or inhibit development of embryos in hamster (Seshagiri & Bavister, 1989; McKiernan *et al*., 1991), sheep (Gardner *et al*., 1993), cattle (Kim *et al*., 1993; Khurana & Niemann, 2000) and mouse (Leese &

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Barton, 1984; Chatot *et al*., 1989). In contrast, glucose has been used in pig embryo culture media without deleterious effects, even when embryos were exposed to 5.6 mM glucose in early stages. Pyruvate and lactate have also been used as energy substrates in pig embryo culture media. Furthermore, it was recently shown that fructose could support embryo development to the blastocyst stage in hamster (Ludwig *et al*., 2001) and cattle (Kwun *et al*., 2003). In previous studies, varying concentrations of glucose (0–5.6 mM), pyruvate (0–0.5 mM) and lactate (0–23 mM) in media such as NCSU-23 (Petters & Wells, 1993), modified Whitten medium (Whitten & Biggers, 1968), Beltsville embryo culture medium-3 (Dobrinsky *et al*., 1996) and Tyrode's lactate-Hepes-glucose medium (Hagen *et al*., 1991) were used for the culture of preimplantation pig embryos. There is limited information on the effect of fructose, and of lactate and pyruvate at different concentrations in the medium. Some workers attempted to improve the composition of energy substrates in culture media (Iwasaki *et al*., 1999; Kikuchi *et al*., 2002) but results were inconsistent, possibly because of differences in embryo origin (*in vivo* versus *in vitro)*, type of medium and culture system (static versus two-step).

This study was conducted to identify energy substrates and concentrations that, when added to serum-free NCSU-23 medium, would support *in vitro* development of porcine embryos. Glucose, fructose, pyruvate and lactate were added, singly and in combination, to evaluate the effect on development (IVD) to the blastocyst stage of preimplantation pig embryos produced *in vitro*.

Materials and methods

Culture media

Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich (St Louis, MO). The medium for oocyte maturation was tissue culture medium (TCM)-199 with Earle's salts and L-glutamine (Life Technologies, Grand Island, NY), supplemented with 26.2 mM $NaHCO_3$, 3.05 mM glucose, 0.91 mM sodium pyruvate, 0.57 mM L-cysteine, 75 μg/ml kanamycin, 20 ng/ml epithelial growth factor (EGF), 10% (v/v) porcine follicular fluid (pFF) and 2 μg/ml porcine FSH (Folltropin-V, Vetrepharm Canada, Ontario, Canada). Basic media for sperm preparation and *in vitro* fertilization (IVF) were phosphate-buffered saline (PBS) and modified Tris-buffered medium (mTBM), respectively, which also were used in previous studies (Wang *et al*., 1997; Abeydeera *et al*., 1998). PBS for sperm washing was supplemented with 0.1% (w/v) bovine serum albumin (BSA; A-9418), 5.6 mM glucose and 0.5 mM sodium pyruvate. Modified TBM consisted of 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2$, 20 mM Tris, 11 mM glucose, 5 mM sodium pyruvate and 0.8% (w/v) BSA (A-3311). The medium for embryo development was a glucose-free NCSU-23 medium containing 0.4% (w/v) BSA, supplemented with energy substrates according to the experimental design.

Oocyte recovery and *in vitro* maturation

Pig ovaries were collected from a local slaughterhouse and transported to the laboratory in 0.9% (w/v) NaCl solution at 30–35 °C. After washing ovaries three times, cumulus–oocyte complexes (COCs) were aspirated from superficial follicles 3–8 mm in diameter with an 18-gauge needle attached to a 5 ml disposable syringe. COCs enclosed by compact cumulus cells with more than three layers and with evenly granulated ooplasm were selected and washed in maturation medium. Then, 55–65 COCs were transferred into each well of a 4-well culture dish containing 500 μl of maturation medium and incubated in a humidified atmosphere of 5% CO_2 in air at 39 °C. After 22 h of maturation culture, oocytes were washed twice, transferred into the same medium without hormonal supplements and cultured for an additional 22 h.

Sperm preparation and IVF

Frozen-thawed boar semen was used for IVF as previously described (Yoon *et al*., 2000). A semen straw was thawed for 1 min in 39 °C water and washed twice by centrifugation at 350 *g* for 3 min in PBS. At the end of the washing, the final sperm pellet was resuspended in mTBM to a concentration of 7.5×10^6 spermatozoa/ml.

Oocytes cultured for maturation were partially denuded by gentle pipetting in a maturation medium containing 0.1% (w/v) hyaluronidase. Fifteen to 20 oocytes in 5 μl of mTBM were placed in a 40 μl drop of fertilization medium covered with warm mineral oil. Then, 5 μl of sperm suspension was added to each fertilization drop to a final sperm concentration of 7.5×10^5 spermatozoa/ml. Gametes were incubated in 5% CO_2 in a humidified air atmosphere for 6 h.

In vitro culture for embryo development

At 6 h following IVF, oocytes were freed from cumulus cells and spermatozoa by repeated pipetting. Presumptive zygotes were washed three times in culture medium, and 8–10 embryos were placed in 30 μl microdrops of appropriately supplemented NCSU-23 medium under mineral oil. Embryos were cultured for 150 h in a humidified atmosphere of 5% CO_2, 7% O_2 and 88% N_2, and embryo development to the blastocyst stage was evaluated at 156 h after IVF under a stereomicroscope (SZ-6045, Olympus, Tokyo, Japan). Sperm penetration and blastomere

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number in blastocysts were examined at 12 h and 156 h after IVF, respectively. At the time of examination, oocytes or blastocysts were mounted on a glass slide and fixed for 10 min in 25% (v/v) acetic acid in ethanol. Fixation was done at 33 °C on a warm plate to remove lipids within 10 min. Slides were stained with 1% (w/v) orcein in 45% (v/v) acetic acid solution and examined for nuclear maturation, sperm penetration, male pronucleus (MPN) formation, or blastomere number in blastocysts under a phase-contrast microscope (Axiphot, Carl Zeiss, Germany) at ×400.

Experimental design

The occurrence of first cleavage division, the occurrence of blastocyst formation and the number of blastocyst cells were recorded as indicators of embryo development. Experiment 1 was conducted to determine which energy substrates, as a single source, would support embryo development. Effects of glucose (5.6 mM), fructose (5.6 mM), pyruvate (0.5 mM) and lactate (10 mM) supplementation were examined. Concentrations were based upon published work with bovine and porcine embryos (Petters & Wells, 1993; Kwun *et al*., 2003; Nguyen *et al*., 2003). In experiment 2, the effect on embryo development of combined additions of glucose and pyruvate, glucose and lactate, pyruvate and lactate, and glucose, pyruvate and lactate were compared. In experiment 3, dose responses of embryo development to pyruvate (0.25, 0.5, 1 or 2 mM) and to lactate (1, 5, 10 or 20 mM) as single energy sources were compared. Based upon results from experiments 2 and 3, a final comparison was made between conventional and a newly formulated NCSU-23 medium in experiment 4. The osmolarities of the media supplemented with various carbohydrates were between 280 ± 3 mOsm in the base medium and 294 ± 4 mOsm in the medium supplemented with 20 mM lactate.

Statistical analysis

Data were analysed by ANOVA using general linear model procedures in the Statistical Analysis System (SAS, version 8.2; Statistical Analysis System Institute, Cary, NC), followed by the least significant difference (LSD) mean separation procedure when treatments differed at $p < 0.05$. Percentage data were subjected to arcsine transformation before analysis to maintain homogeneity of variance. Results are expressed as mean ± standard error of the mean (SEM).

Results

Averaged across all experiments, nuclear maturation and sperm penetration averaged were 91 ± 3% and 69 ± 4%, respectively. Monospermic fertilization and MPN formation were found in 59 ± 4% and 81 ± 3% of the penetrated oocytes, respectively.

Table 1 Effects of the addition of single energy substrates to a serum-free culture medium on cleavage, blastocyst formation and cell number of porcine embryos following *in vitro* maturation and fertilization

Addition of:	No. of embryos cultured	% of embryos developed to:		Cell number/ blastocyst
		2-cell	Blastocyst	
None	110	13 ± 3^{a}	0^{a}	–
Glucose, 5.6 mM	139	53 ± 8^{b}	20 ± 4^{bc}	25 ± 2^{a}
Fructose, 5.6 mM	137	10 ± 2^{a}	0^{a}	–
Pyruvate, 0.5 mM	140	63 ± 8^{b}	27 ± 2^{b}	32 ± 3^{ab}
Lactate, 10 mM	143	63 ± 11^{b}	18 ± 3^{c}	38 ± 4^{b}

^{a,b,c} Values with different superscripts are significantly different, $p < 0.05$.

Experiment 1: Effects of the addition of single carbohydrates

Energy sources differed significantly ($p < 0.05$) in their impact on all measures of embryonic developmental competence. As shown in Table 1, the single addition of glucose, pyruvate or lactate significantly improved embryo cleavage (53–63% vs 10–13%, $p < 0.01$) and blastocyst formation (18–27% vs 0, $p < 0.01$), compared with no energy supplementation or the addition only of fructose. Blastocyst formation was not possible with no energy supplementation or following the single addition of fructose. The addition of lactate significantly increased the number of blastocyst cells compared with the addition of glucose (38 cells vs 25 cells, $p < 0.05$), while no difference in cell number was found following lactate or pyruvate addition (38 and 32 cells, respectively).

Experiment 2: Effects of single and combined additions of glucose, pyruvate and lactate

Treatments differed ($p < 0.01$) in their impact on blastocyst formation but not on cleavage ($p = 0.66$) or blastocyst cell number ($p = 0.10$). As shown in Table 2, joint supplementation with pyruvate and lactate significantly improved blastocyst formation (25%) compared with other treatments except for the single addition of pyruvate or glucose. The addition of pyruvate and glucose significantly ($p < 0.01$) inhibited blastocyst formation compared with the single addition of glucose. Blastocyst cell number was within the range of 23 to 40 cells for all treatments, with no statistically significant differences.

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Table 2 Effects of the addition of single or combined energy substrates to a serum-free culture medium on cleavage, blastocyst formation and cell number of porcine embryos following *in vitro* maturation and fertilization

Addition of:	No. of embryos cultured	% of embryos developed to: 2-cell	Blastocyst	Cell number/ blastocyst
G	97	50 ± 4	18 ± 3^{ab}	25 ± 3
P	142	64 ± 8	22 ± 3^{ab}	30 ± 3
L	140	53 ± 4	15 ± 4^{bc}	40 ± 5
G + P	142	53 ± 7	9 ± 3^{cd}	26 ± 3
G + L	139	58 ± 11	14 ± 2^{bc}	33 ± 4
P + L	141	66 ± 9	25 ± 5^{a}	33 ± 3
G + P + L	142	60 ± 6	6 ± 2^{d}	23 ± 4

G, 5.6 mM glucose; P, 0.5 mM pyruvate; L, 10 mM lactate.
[a,b,c,d] Values with different superscripts are significantly different, $p < 0.01$.

Table 3 Effects of pyruvate concentration in a serum-free culture medium on cleavage, blastocyst formation and cell number of porcine embryos following *in vitro* maturation and fertilization

Concentrations (mM)	No. of embryos cultured	% of embryos developed to: 2-cell	Blastocyst	Cell number/ blastocyst
0.25	160	41 ± 4	13 ± 2	53 ± 6^{a}
0.5	157	52 ± 6	17 ± 3	39 ± 5^{ab}
1.0	156	51 ± 8	10 ± 2	30 ± 5^{b}
2.0	153	48 ± 6	10 ± 3	32 ± 6^{b}

[a,b] Values with different superscripts are significantly different, $p < 0.05$.

Experiment 3: Dose response of embryo developmental competence to pyruvate and lactate concentration

As shown in Table 3, no significant differences in cleavage or in blastocyst formation rate were found among pyruvate concentrations (0.25–2 mM). Blastocyst cell number decreased as pyruvate concentration increased. On the other hand, lactate concentration did affect blastocyst formation ($p = 0.001$). As shown in Table 4, a higher percentage of embryos developed to the blastocyst stage (14–18% vs 7%) after the addition of 5, 10 or 20 mM than after the addition of 1 mM lactate ($p < 0.05$). There was no significant difference in blastomere cell number among concentrations (32 – 37 cells).

Experiment 4: Effectiveness of a newly designed medium containing pyruvate and lactate

Based on the results of experiments 2 and 3, our standard culture medium was reformulated by replacing the 5.6 mM glucose supplementation to serum-free NCSU-23 medium with 0.5 mM pyruvate and/or 5 mM lactate. The combined addition of pyruvate and lactate improved blastocyst formation (32% vs 18%; $p = 0.05$) compared with the single addition of glucose but this treatment was not significantly different from the substitution of glucose by pyruvate or by lactate alone (Table 5).

Table 4 Effects of lactate concentration of a serum-free culture medium on cleavage, blastocyst formation and cell number of porcine embryos following *in vitro* maturation and fertilization

Concentrations (mM)	No. of embryos cultured	% of embryos developed to: >2-cell	Blastocyst	Cell number/ blastocyst
1	177	35 ± 6	7 ± 1^{a}	35 ± 5
5	183	45 ± 10	17 ± 3^{b}	34 ± 3
10	176	46 ± 6	14 ± 1^{b}	32 ± 4
20	171	45 ± 7	18 ± 2^{b}	37 ± 6

[a,b] Values with different superscripts are significantly different, $p < 0.05$.

Table 5 Effect of conventional (G) or newly designed energy supplementations to a serum-free medium on cleavage, blastocyst formation and cell number of porcine embryos following *in vitro* maturation and fertilization

Energy substrate (mM)	No. of embryos cultured	% of embryos developed to: 2-cell	Blastocyst	Cell number/ blastocyst
G (5.6)	216	45 ± 1	17 ± 2^{a}	46 ± 4
L (5.0)	221	47 ± 5	21 ± 3^{ab}	46 ± 3
P (0.5)	216	53 ± 8	23 ± 0^{ab}	40 ± 3
P (0.5) + L (5.0)	211	53 ± 5	28 ± 4^{b}	45 ± 3

G, glucose; P, pyruvate; L, lactate.
[a,b] Values with different superscripts are significantly different, $p < 0.05$.

Discussion

Results of this study establish that pyruvate and/or lactate can replace conventional glucose supplementation to NCSU-23 medium to support *in vitro* development of porcine embryos. Blastocyst formation rate was 32% from culturing embryos in BSA-containing NCSU-23 medium supplemented with 0.5 mM pyruvate and 5 mM lactate. Interestingly, fructose supplementation and the combined supplementation of glucose and pyruvate inhibited pig embryo development. Apparently, the composition and concentration of energy substrate added to a serum-free culture

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medium is critical for embryo development, and requirements for porcine embryos differ from those of other species.

Porcine (Flood & Weibold, 1988; Swain *et al.*, 2002), bovine (Khurana & Niemann, 2000) and murine embryos (Leese & Barton, 1984) differ in glucose metabolism and may differ in energy requirements as well. This hypothesis was confirmed by our results showing that neither glucose nor fructose promoted blastocyst in pig embryos whereas they do support IVD of bovine and hamster embryos (Ludwig *et al.*, 2001; Kwun *et al.*, 2003). Our results further suggest that pyruvate and lactate, as a replacement for glucose, can improve embryo development to the blastocyst stage. These results suggest that energy supplementation to culture media should be separately formulated for each species, to optimize conditions for embryo development and growth.

Glucose supplementation in the presence of pyruvate significantly inhibited pig embryo development in our experiment, but this was not found in cattle (Rieger *et al.*, 1992; Takahashi & First, 1992). Iwasaki *et al.* (1999) also reported that pyruvate and glucose in combination significantly inhibited pig embryo development to the morula stage. Species-specific requirements of exogenous glucose and pyruvate and metabolic alteration among species were not directly elucidated in this study. In another report, it was reported that glucose can be metabolized into pyruvate through a glycolytic pathway (Swain *et al.*, 2002), and pyruvate can be converted into lactate. It was not known in this study whether the inhibitory effect of glucose and pyruvate was attributed to direct interaction between the two carbohydrates or to metabolic outcome from glucose and pyruvate metabolism. In contrast to our results in the pig, Kwun *et al.* (2003) reported that culture medium supplemented with 5.6 mM fructose supported bovine embryo development more effectively than the same medium supplemented with 5.6 mM glucose. Further studies are needed to clarify such an inhibitory action.

Lactate and pyruvate are present in pig oviductal fluid at concentrations of 5.71 mM and 0.21 mM, respectively (Nichol *et al.*, 1992). Lactate inhibits preimplantation embryo development in the porcine (Davis & Day, 1978; Davis, 1985), and pyruvate does not support development to the blastocyst stage (Petters & Reed, 1991). In our experiment, lactate and pyruvate had beneficial rather than inhibitory effects, which is consistent with findings (Kikuchi *et al.*, 2002; Yoshioka *et al.*, 2002) that pyruvate and lactate in a glucose-free medium result in a high rate of blastocyst formation of pig embryos. It is unclear how lactate and pyruvate improve embryo development *in vitro*. Lactate production is increased as embryos develop (Gandhi *et al.*, 2001), and a higher concentration of lactate than glucose and pyruvate is present in pig oviductal fluid. Pyruvate is also detected in pig oviductal fluid and may act as an antioxidant (O'Fallon & Wright, 1995). Pyruvate and lactate may regulate intracellular pH and protect embryos from the oxidative stress within the culture environment during IVD.

In our results, there was no significant difference in embryo development among pyruvate concentrations (0.25–2 mM), while a lactate concentration lower than 5 mM greatly inhibited cleavage and blastocyst formation. These results suggest, to some extent, that higher concentrations of pyruvate and lactate in the culture medium than in oviductal fluid are not harmful to the growth of pig embryos. Recently, Nguyen *et al.* (2003) reported in parthenogenetic pig embryos that a change from isotonic (280–310 mOsm) to hypotonic (220–270 mOsm) medium at 48–72 h after electric activation enhanced embryo cleavage and blastocyst formation. Although the osmolarities of media tested in this study ranged from 280 to 294 mOsm and medium was not changed through the entire culture period, we could not exclude the probable effect of different osmolarities.

In this study, our standard pig embryo culture medium was altered by replacing glucose with 0.5 mM pyruvate and 5 mM lactate. Pyruvate concentration in this medium was higher than that in pig oviductal fluid (Nichol *et al.*, 1992) and in other media used for pig embryo culture (Kikuchi *et al.*, 2002; Yoshioka *et al.*, 2002). Lane & Gardner (2000) reported that pyruvate uptake is inhibited when lactate is also present in a medium. Pyruvate can act as an antioxidant in mouse embryo development (O'Fallon & Wright, 1995). Although blastocyst formation and blastomere number of embryos did not differ as pyruvate concentration varied from 0.25 mM to 0.5 mM in this study, the 0.5 mM concentration was selected due to the potential benefit of the higher concentration to balance decreased pyruvate uptake due to lactate and in protecting embryos from oxidative stress.

Blastocyst formation rate was improved in NCSU-23 medium supplemented with pyruvate and lactate, compared with the conventional medium containing glucose. In contrast to these results, Kikuchi *et al.* (2002) did not show improvement in blastocyst development on lactate and pyruvate supplemented media, compared with media with glucose supplementation, in a study in which oocytes were matured in 5% O_2 and cultured in NCSU-37 medium. It is difficult to compare their findings directly with ours, because differences in cultural conditions and environment can modulate embryo metabolism. The medium developed in this study was shown to be effective for pig embryo development. A previous finding (Yoshioka *et al.*, 2002) that more pig blastocysts could be obtained from a medium containing pyruvate and lactate, but

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not from a medium containing glucose, indirectly confirmed the applicability of our developed medium. However, further research is necessary to evaluate effects of the medium on post-transfer embryo viability and embryo implantation. Furthermore, it should be determine whether this medium affects development of reconstructed oocytes following nuclear transfer.

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