—Research Note—

Effect of Replacement of Pyruvate/Lactate in Culture Medium with Glucose on Preimplantation Development of Porcine Embryos *In Vitro*

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Abstract. The effect of glucose supplementation at different times in *in vitro* culture on the developmental competence of *in vitro* produced (IVP) porcine embryos was examined. In Experiment 1, when IVP embryos were cultured in modified NCSU-37 supplemented with pyruvate and lactate (IVC-pyr/lac) for 0 h, 24 h, 48 h, 72 h, 96 h, or 118 h and subsequently in modified NCSU-37 supplemented with glucose (IVC-glu) until Day 6 (Day 0=day of *in vitro* fertilization), the rates of blastocyst formation were significantly higher in embryos cultured in IVC-pyr/lac for 24 or 48 h (24.4% and 23.0%, respectively) than in embryos cultured in IVC-pyr/lac for the whole culture period (14.5%). However, there were no significant differences between embryos obtained after the energy source replacement and embryos cultured in IVC-glu for the whole culture period on the rates (15.2%–24.4%, and 16.8% respectively). Replacement of pyruvate/lactate with glucose at 58 h of culture in Experiment 2 significantly enhanced the rate (31.3%) compared to those after replacement at 48 h, 53 h and 63 h of culture (20.6%, 20.8%, and 21.1%, respectively). In conclusion, replacement of pyruvate/lactate with glucose as the energy substrate was optimal at 58 h of culture for the development of porcine embryos to the blastocyst stage.

Key words: Glucose, Pyruvate, Lactate, Pig embryos, IVC

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The role and effect of glucose as a major energy substrate in the composition of an embryo culture on preimplantation embryo development *in vitro* has been demonstrated in many species. Most reports show that glucose inhibits embryo development before compaction or before the blastocyst stage in mice [1–4] and cattle [5]. In

contrast, glucose is inhibitory to hamster embryo development in all the preimplantation stages [6, 7]. Also it has been suggested that developmental responses of preimplantation embryos to glucose *in vitro* depend both on the stage and interactions with other substrates in the medium [8] and seem to be species-specific [9]. A better understanding of the metabolism or the energy substrate and nutrient preferences of preimplantation embryos

may provide an insight into their metabolic requirements.

Swain *et al.* [9] reported that the metabolism of porcine embryos produced both *in vivo* and *in vitro* is different from that of other species, as they metabolize glucose throughout preimplantation development. Flood and Weibold [10] demonstrated that glucose utilization in porcine embryos produced *in vivo* increases during early development until compaction and blastulation. In contrast *in vitro* development of *in vitro*-derived porcine embryos at the earliest preimplantation stages can be supported by pyruvate and lactate [11–13]. A previous study by us reported that replacement of pyruvate and lactate with glucose, as energy substrate at 48 h of culture enhanced blastocyst formation and the quality of the *in vitro* produced (IVP) porcine blastocysts [13]. Furthermore our most recent study showed that the replacement at 58 h of culture was better than at 46 h for the development of IVP embryos to the blastocyst stage [14]. However, because glycolytic activity of *in vitro*-derived porcine embryos was reported to increase around the eight-cell stage [10], and although there was a wide variety of developmental embryonic stages at Day 2 (Day 0 = day of *in vitro* fertilization) of culture (two- to eight-cell stages with most of them at the late four-cell stage) [13], only replacement at 46, 48 and 58 h after IVC of IVP embryos were compared in those studies. Thus the exact time during the early cleavage stages, when the utilization of glucose as an energy source is optimal, needs to be determined for the effective *in vitro* production of porcine embryos. The purpose of this study was to examine the effects of glucose supplementation at different times of culture on the developmental competence of IVP porcine embryos.

Materials and Methods

Oocyte collection and in vitro maturation (IVM)

Porcine cumulus-oocyte complexes (COCs) were matured and fertilized *in vitro* according to Kikuchi *et al.* [13]. Porcine ovaries were obtained from prepubertal gilts at a local slaughterhouse and transported to the laboratory at 35 °C. COCs were aspirated through an 18-gauge needle into a disposable 10-ml syringe from follicles of 3–6 mm in diameter, and were collected in tissue culture

medium (TCM) 199 with Hanks' salt (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% (v:v) fetal calf serum (Cell and Molecular Technology, USA), 20 mM Hepes (Dojindo Laboratories, Japan), 100 IU/ml penicillin G potassium and 0.1 mg/ml streptomycin sulfate. Only COCs with multilayered compact cumulus cells and evenly granulated cytoplasm were selected. Groups of 40–50 COCs were cultured for 20–22 h in 500 μ l of maturation medium in a four-well dish (Nunc Multidishes; Nalge Nunc International, Denmark). The maturation medium consisted of a modified North Carolina State University (NCSU)-37 solution supplemented with 10% (v:v) porcine follicular fluid, 0.6 mM cysteine, 1 mM dibutyryl cyclic AMP (dbcAMP; Sigma Chemical Co., St. Louis, MO, USA), 50 μ M β -mercaptoethanol (Wako, Japan), 10 IU/ml PMSG (Sankyo, Japan), and 10 IU/ml human chorionic gonadotropin (Sankyo, Japan). They were subsequently cultured in the NCSU-37 solution without dbcAMP and hormones for 24 h. All cultures were performed at 38.5 °C, 5% O₂, 5% CO₂, and 90% N₂.

In vitro fertilization (IVF) and embryo culture

Frozen spermatozoa were thawed and preincubated for 15 min at 38.5 °C in TCM-199 with Earle's salts (Gibco Life Technologies Inc., Grand Island, NY, USA) adjusted to pH 7.8. A portion (10 μ l) of preincubated spermatozoa was introduced into 90 μ l of fertilization medium containing 10–15 oocytes. The fertilization medium consisted of 90 mM NaCl, 12 mM KCl, 25 mM NaHCO₃, 0.5 mM NaH₂PO₄, 0.5 mM MgSO₄, 10 mM sodium lactate, 0.5% bovine serum albumin (BSA; Fraction V; Sigma Chemical Co., St. Louis, MO, USA), and 5 mM caffeine benzonate (Sigma Chemical Co., St. Louis, MO, USA). The final sperm concentration was 1×10^5 /ml. Five hours after co-incubation with spermatozoa, cumulus cells surrounding zygotes were removed by pipetting with a small-bore pipette and cultured in IVC medium. The basic medium for embryo development was modified NCSU-37 medium (mNCSU-37) containing 4 mg/ml BSA and 50 μ M β -mercaptoethanol.

Assessment of the total cell numbers in the blastocyst

The total cell number in the blastocyst at Day 6 (Day 0 = day of IVF) was determined after nuclear staining in the presence of 20 μ g/ml Hoechst 33342

(Sigma Chemical Co., St. Louis, MO, USA) for 15 min at 37 C. Fluorescent staining of the nuclei was visualized with a fluorescence microscope.

Statistical analysis

The percentages of embryos cleaved and developed to the blastocyst stage were subjected to arc sin transformation before analysis, and then tested by a post hoc, Fisher's protected least significant difference test (PLSD test). The mean cell number of the blastocysts was analyzed by Student's t-test analysis. Differences at a probability of $P < 0.05$ were considered significant.

Experimental design

Experiment 1: Experiment 1 (6 trials) was conducted to examine the effect of replacement of pyruvate and lactate with glucose as the energy source at different times of culture on the developmental competence of IVP porcine embryos. After being fertilized (day 0), putative zygotes (1158 in total) were cultured in mNCSU-37 (500 ml) supplemented with 0.17 mM sodium pyruvate and 2.73 mM sodium lactate (IVC-pyr/lac) in 4-well dishes. Embryos (30–50 in each group) were then transferred into mNCSU-37 supplemented with 5.55 mM D-glucose (IVC-glu) at 24 h, 48 h, 72 h, 96 h, or 118 h of culture. As control groups, putative zygotes were cultured in IVC-pyr/lac or IVC-glu for the whole culture period (IVC-pyr/lac-only and IVC-glucose-only, respectively).

Experiment 2: Because glycolytic activity in *in vitro*-derived porcine embryos was reported to increase around the eight-cell stage and some embryos developed to that stage before 72 h of culture in Experiment 1, Experiment 2 (4 trials) was conducted to examine the effect of replacement of pyruvate and lactate with glucose as the energy source at 48 to 63 h of culture on the developmental competence of IVP porcine embryos. After being fertilized, putative zygotes (543 in total, and 30–50 zygotes in each group) were cultured in IVC-pyr/lac, and then transferred into IVC-glu at 48 h, 53 h, 58 h, or 63 h of culture.

All the embryos were cultured until Day 6, and the development to the blastocyst stage and number of cells per each blastocyst were then assessed.

Results

When IVM/IVF embryos were cultured in IVC pyr/lac for 24 h or 48 h and subsequently in IVC-glu until Day 6, the rate of blastocyst formation (24.4% and 23.0%, respectively) was significantly higher ($P < 0.05$) than that of IVC-pyr/lac-only group (14.5%) (Table 1). However, when IVC pyr/lac was replaced with IVC-glu, there was no significant difference between the energy source replacement groups and the IVC-glucose-only group in terms of the proportions of cleavage, development to the blastocyst stage and mean cell number per each blastocyst (Table 1).

As shown in Table 2, replacement of pyruvate and lactate with glucose in the culture medium at 58 h of culture significantly enhanced the rate of blastocyst formation compared with zygotes cultured in the medium where the replacement was done at 48 h, 53 h and 63 h of culture (31.3% vs 20.6%, 20.8%, and 21.1%, respectively ($P < 0.05$)). However, no effect on the mean cell number was observed.

Discussion

The results of the present study demonstrated that in the absence of glucose, the development rate of embryos cultured in the medium with pyruvate/lactate, as the energy source, for the whole culture period was significantly lower than those for embryos cultured in the presence of pyruvate/lactate in combination with glucose. Replacement of pyruvate/lactate with glucose as the energy source enhanced the preimplantation development of porcine embryos and a significantly higher rate of blastocyst formation was obtained in the presence of glucose at 24 h to 48 h of culture compared to without the replacement. It has been reported that embryo metabolism is biphasic; at the time of embryonic activation, energy metabolism switches from the use of lactate and pyruvate via the Krebs cycle and oxidative phosphorylation to the primary use of glucose via the Embden-Meyerhof pathway [15, 16]. With regard to this, it has been shown that the absence of glucose in the later preimplantation stages of bovine embryos caused a decrease in the blastocyst total cell number [15]. Kikuchi *et al.* [10] and Medvedev *et al.* [14] demonstrated that pyruvate and lactate are

Table 1. Effect of replacement of pyruvate/lactate in culture medium with glucose at 24 to 118 h of culture on preimplantation development of porcine embryos *in vitro*

Culture medium		No. of oocytes examined	No (%) of oocytes cleaved	No. (%) of embryos develop to blastocysts	Mean $\pm$ SEM of total cell number in blastocyst
Pyr/lac (h)	Glucose (h)				
0–144	–	234	136 (58.1)	34 (14.5) ^a	34.0 $\pm$ 12.8
–	0–144	232	145 (62.5)	39 (16.8) ^{a,b}	37.3 $\pm$ 15.9
0–24	24–144	234	150 (64.1)	57 (24.4) ^b	42.1 $\pm$ 17.2
0–48	48–144	235	140 (59.6)	54 (23.0) ^b	35.8 $\pm$ 15.1
0–72	72–144	223	141 (63.2)	48 (21.5) ^{ab}	35.7 $\pm$ 10.4
0–96	96–144	246	151 (61.4)	46 (18.7) ^{ab}	39.6 $\pm$ 18.3
0–118	118–144	145	93 (64.1)	22 (15.2) ^{ab}	39.3 $\pm$ 10.3

^{a,b}Values with different superscripts within the same column are significantly different ($P < 0.05$).

Table 2. Effect of replacement of pyruvate/lactate in culture medium with glucose at 48 to 64 h of culture on preimplantation development of porcine embryos *in vitro*

Culture medium		No. of oocytes examined	No (%) of oocytes cleaved	No. (%) of embryos develop to blastocysts	Mean $\pm$ SEM of total cell number in blastocyst
Pyr/lac (h)	Glucose (h)				
0–48	48–144	131	80 (61.1)	27 (20.6) ^a	38.8 $\pm$ 17.0
0–53	53–144	136	84 (61.2)	28 (20.8) ^a	40.7 $\pm$ 9.4
0–58	58–144	134	89 (66.4)	42 (31.3) ^b	40.6 $\pm$ 15.7
0–63	63–144	142	91 (64.1)	30 (21.1) ^a	42.5 $\pm$ 13.6

^{a,b}Values with different superscripts within the same column are significantly different ($P < 0.05$).

important energy supplements in porcine, especially for an early embryonic development *in vitro*, while blastocyst formation requires much energy due to increased protein synthesis levels [17] and to increased activity of the Na^+/K^+ -ATPase to create the blastocoel cavity [18].

Since there was a tendency for more embryos to develop to the blastocyst stage in the presence of glucose at 24 h to 72 h of culture in the present study, the glycolytic activity in IVP porcine embryos seemed to increase after the eight-cell stage [9]. Also the first two days after IVF were suggested to be the prerequisite stage for embryonic genome activation [19], at which stage a four-cell block was observed in porcine [13]. Thus, it is probable that an adequate energy source would enhance the embryonic potential for genomic activation or for overcoming the developmental block. Exposure to glucose starting from 48 h to 63 h of culture in the present study (Experiment 2), showed that replacement of pyruvate/lactate with glucose at 58 h of culture, permitted a greater number of embryos to develop to the blastocyst stage, suggesting that porcine IVP embryos can

effectively utilize glucose as energy starting at around 58 h of culture. Our results were consistent with those reported by Medvedev *et al.* [14] in which the developmental potency of IVP porcine embryos and the quality of blastocysts obtained depended on when exposure to glucose began. Exposure to glucose starting at 58 h of culture facilitated the embryos to develop to full blastocysts, but exposure at an earlier stage resulted in roughly half the rate of full blastocyst formation and a substantial increase in the incidence of malformed and dead embryos [14].

When porcine embryos were cultured in the presence of glucose during the whole culture period in the present study, the developmental competence to the blastocyst stage was similar to those found for embryos cultured in the presence of pyruvate/lactate prior to being cultured in glucose supplemented medium. However, although there were no significant differences between the glucose-only group and the energy source replacement groups in the developmental rate, the rates of embryos cultured in the presence of glucose during the whole culture period tended to be lower

than those for embryos that were cultured in the presence of pyruvate/lactate in combination with glucose, and a similar phenomenon was observed in embryos cultured with pyruvate/lactate during the whole culture period. Our results suggest that glucose utilization during the preimplantation development in porcine embryos follows the pattern observed by Flood and Wielbold [10]. Levels of utilization were relatively low at the early stages, but increased steadily with development. Porcine embryos have been reported to be able to metabolize glucose throughout preimplantation development, and glucose contributed as an energy source [9, 20]. At the early stages of development (1–4 cell stages), porcine embryos utilize glucose via the pentose phosphate pathway [10]. During the later stages of development, glucose utilization is mostly via the glycolytic pathway. However, it is known that exposure of embryos to high concentrations of glucose during the early

embryonic stages causes developmental retardation in many species [4–6, 17]. Kikuchi *et al.* [13] and Medvedev *et al.* [14] suggest that glucose supplementation in early development might cause harmful effects, such as oxidative stress on genome activation or difficulty in overcoming the four-cell block at around Day 2 of IVC in porcine. The toxicity of methylglyoxal, a metabolic by-product of glycolysis has been reported to cause inactivation of the intracellular glutathione peroxidase that is responsible for scavenging free oxygen radicals [21]. Perhaps this enzyme is minimally active in early porcine embryos, with a substantial increase in activation at approximately 58 h of culture [14].

Replacement of pyruvate and lactate with glucose as the energy substrate in the culture medium was optimal at 58 h of culture for the development of pig embryos to the blastocyst stage.

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