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Inhibitory Effect of Iodoacetate on Developmental Competence of Porcine Early Stage Embryos *In Vitro*

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In porcine preimplantation embryos, glucose-utilizing pathways are reported active throughout their development. The aim of this study was to test the involvement of glycolysis activity in early stage of porcine embryo development by the addition of iodoacetate, a glycolytic inhibitor, to culture medium. *In vitro* matured and fertilized oocytes were cultured for the first 2 days in iodoacetate at concentrations of 0, 1, or 2.5 µM. The proportion of viable embryos on day 4 decreased when Ia was added at concentration of 2.5 µM ($P < 0.05$). The development of cleaved embryos to the blastocyst stage on day 6 was significantly reduced by 1 µM iodoacetate and none of cleaved embryos in Ia-2.5 group develop to the blasyocyst stage ($P < 0.05$). More than 60% of embryos in Ia-2.5 group were arrested at the two-four-cell stage, and then arrested at the morula stage. These results indicate that glucose plays important roles in supporting the development of early stage of porcine embryos and that the inhibition of glucose metabolism may disrupt the management of energy production, leaving the embryos incompetence to develop.

Key words: iodoacetate, glucose, early stage embryos, *in vitro* culture

INTRODUCTION

The growth and development of preimplantation embryos requires considerable metabolic activity for the production of energy and synthesis of a variety of complex molecules. Mammalian preimplantation embryo undergoes significant changes to generate energy between zygote and blastocyst stages (Gardner 1998). In most mammalian embryos, metabolic activity and substrates preferences of embryos appear to switch from tricarboxylic acid cycle dependence during the early cleavage stages to glycolysis at the post-compaction stage (Houghton *et al*. 1996). Metabolic activity of the early embryo resembles that of mature oocyte; significant metabolism of pyruvate and glutamine but little or no metabolism of glucose (Rieger *et al*. 1992, 1996). Pyruvate and glutamine metabolism then decline while there is a significant increase in glucose metabolism coincident with the activation of the embryonic genome (Rieger 1996). Thus, the presence of glucose in culture medium in concentrations within normal physiological range universally inhibit early embryonic development of a number of species (Bavister 1995) and this effect may be mediated by changes in the activity of glycolytic enzymes (Rieger 1996).

In pigs, on the other hand, the metabolic activity of the embryos has been reported differ from that of other species. Nutrient uptake studies carried out on porcine embryos have shown that porcine embryos consume glucose and produce lactate at all stages of development, suggesting that glucose-utilizing pathways are active throughout embryonic development, and that glucose does contribute as an energy source (Flood & Wiebold 1988; Petters *et al*. 1990; Swain *et al*. 2002; Kikuchi *et al*. 2002; Karja *et al*. 2006). Likewise, study on the metabolic regulation of in vitro derived porcine embryos demonstrated that different glucose utilizing pathways may be more or less active during different periods of development. Glucose metabolism via pentose phosphate pathway was higher at zygotes and two-cell stage; whereas, glycolytic activity was found to occur throughout their development and increased after the eight-cell stage of development (Gandhi *et al*. 2001; Swain *et al*. 2002; Sturmey & Leese 2003).

The present study was carried out to examine the role of glycolytic activity on glucose metabolism of early developmental stages of porcine embryos *in vitro* by the addition of iodoacetate (Ia). Iodoacetate is an inhibitor of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a glycolytic enzyme that catalyses the conversion of glyceraldehyde-3-phosphate to 1,3-biphosphoglycerate resulting in production of NADH. Although the effects of substrate on embryo development in pigs have been examined to provide clues about metabolism, few studies have tried to block metabolism with inhibitors to assess responsible pathways. Work with inhibitors may help explain some of the available data on substrate specificity by indicating at what stages of development changes in metabolic pathways occur and where differences exist among species.

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MATERIALS AND METHODS

***In Vitro* Maturation (IVM) and *In Vitro* Fertilization (IVF) of Porcine Oocytes.** The procedures for oocyte collection, IVM, and IVF have been described previously by Karja *et al*. (2004) with minor modifications. Porcine cumulus-oocyte complexes (COCs) were collected from 2-6 mm follicles of slaughterhouse ovaries and oocytes with intact cumulus cells and an evenly granulated cytoplasm were used in this experiment. About 50 COCs were cultured for 20-22 h in 500 µl of maturation medium, a modified North Carolina State University (NCSU)-37 solution (Petters & Wells 1993) supplemented with 10% porcine follicular fluid, 0.6 mM cysteine, 1 mM dibutyryl cyclic AMP (dbcAMP; Sigma), 50 µM â-mercaptoethanol, 10 IU/ml pregnant mare serum gonadotropin, 10 IU/ml human chorionic gonadotropin, and 50 µg/ml gentamicin. They were subsequently cultured in the NCSU-37 solution without hormones for 24 h. All culture was performed at 38.5 ^{o}C and 5% CO_2 under 5% O_2 and 90% N_2.

At the end of maturation culture, spermatozoa were thawed and preincubated for 15 min at 38.5 ^{o}C in a tissue culture medium (TCM) 199 with Earle's salts adjusted to pH 7.8. A portion (10 µl) of preincubated spermatozoa was introduced into 90 µl of fertilization medium containing 10-20 matured COCs. The oocytes were coincubated with spermatozoa for 3 h. Presumptive zygotes were obtained after 3 h of IVF and cumulus cells were removed by pipetting vigorously. They were subsequently cultured randomly in groups of ten per 100 µl of the media to which they had been designed. The day of IVF was defined as day 0.

The base medium used for embryo culture in present study was NCSU-37 containing 4 mg/ml BSA and 50µM â-mercaptoethanol and 5.5 mM glucose (IVC medium). Treatments were prepared by modifying the base medium to contain various concentration of sodium iodoacetate (Ia, Sigma) as described below.

Effect of Glycolysis Inhibitor on Development of *In Vitro* Produced (IVP) Porcine Embryos. To determine the effect of iodoacetate, a glycolytic activity inhibitor, during the first 2 days of culture on development of porcine embryos to the blastocyst stage *in vitro*, presumptive zygotes were cultured from days 0 to 2 in IVC medium containing 1, 2.5, or 5 ìM iodoacetate (Ia-1, -2.5, -5, groups, respectively). The embryos then were continuously cultured until day 6 in IVC medium only. As Control group, presumptive zygotes were culture in IVC medium only. Since at concentration of 5 µM, iodoacatete completely inhibit first cleavage division of embryos, hence in the next experiment this group was not tested.

For examination of the embryo development, on day 6 all embryos in experiment 1 were fixed and permeabilized for 15 min at room temperature with 3.7% paraformaldehyde in PBS containing 1% (v/v) Triton X-100 (Sigma), and then placed in PBS supplemented with 0.3% (w/v) polyvinylpyrrolidone (PVP; Sigma) for 15 min at room temperature. The embryos were placed on a microslide in a drop of mounting medium consisting of 90% (v/v) glycerol containing 1.9 µM bis-benzimide (Hoechst 33342; Calbiochem; EMD Biosciences, Inc. La Jolla, CA, USA.). A coverslip was placed on top of the embryos and the staining of the nuclei was examined with a fluorescence microscope (BX 51; Olympus, Tokyo). The numbers of cleaved embryos including the stage of embryo development, blastocysts rate, and total cell number of blastocyst were recorded.

Embryo Evaluation with Live-Dead Nuclear Staining. The viability of the embryos after culturing with iodoacetate for the first 2 days of culture was assessed using fluorescein diacetate (FDA) as described by Somfai *et al*. (2006) with minor modifications. Fluorescein diacetate, a non polar ester, passes through cell membranes and is hydrolyzed by intracellular esterases to produce fluorescein, a polar compound that accumulates inside live cells and emits green fluorescein. After culturing the embryos for 2 days with 1 or 2.5 iodoactete, the embryos were then continuously cultured for further 2 days in IVC medium only. On day 4, all cleaved embryos in each group were incubated for 15 min in PBS supplemented with 5 mg/ml BSA and 1 µg/ml fluorescein diacetate (FDA; Sigma), 50 µg/ml propidium iodide (PI; the cell-impermeant dye; Sigma), and 20 µg/ml Hoechst 33342 (the cell-permeant dye). The embryos were then washed in PBS-BSA and immediately mounted on glass slides covered with a coverslip. They were examined under UV light with an epifluorescence microscope (BX- 51, Olympus, Tokyo, Japan). Viable embryos displayed a positive bright green fluorescence throughout ooplasm with blue nuclei (labeled with Hoechst only but not PI), whereas dead embryos showed no green fluorescence with nuclei labeled with both Hoechst and PI, thus appeared red. The embryos with partly displayed green fluorescence blastomere with red nuclei were considered to be partly viable embryo.

Statistical Analysis. Data were expressed as means $\pm$ SEM. The percentages of embryos that developed to the blastocyst stage and the percentages of embryos viability were subjected to arcsine transformation before analysis variance (ANOVA). The transformed data were tested by ANOVA followed by post hoc testing with Fisher's protected least significant difference test (PLSD test) using Statview program (Abacus concepts, Inc., Berkeley, CA, USA). Differences with a probability value (P) of 0.05 or less were considered significant.

RESULTS

Culturing of porcine zygotes with up to 2.5 µM iodoacetate for the first 2 days of culture had no significant effect on the cleavage rate and total cell number in blastocysts, as compared with control ($P > 0.05$) (Table 1). However, the evaluation of final developmental stages of embryos at day 6 showed that development of cleaved embryos to the blastocyst stage was significantly reduced by 1 µM Ia ($P < 0.01$), whereas none of cleaved embryos develop to the blastocyst stage when cultured with 2.5 µM Ia. Developed porcine embryos at blastocyst stage (day 6) was represented in Figure 1. A higher proportion of cleaved embryos in Ia-1 group were arrested at morula stage than that of the control group ($P < 0.05$). More than 60 % of embryos in Ia-2.5 group

Table 1. Development competence of *in vitro* produced porcine embryos after culturing in different concentration of iodoacetate (Ia) for the first 2 days of culture*

Iodoacetate (Ia) concentration	No. of zygotes examined	No. (%) of embryos cleaved on day 2	No. (%) of final developmental stages of cleaved embryos** on day 6					Total cell number in blastocyst
			2-cell	3-4-cell	6-8-cell	Morula	Blastocyst	
0 (control)	180	78 (43.3 ± 4.6)	6 (8.0 ± 2.1)a	12 (15.6 ± 4.0)a	12 (14.0 ± 4.2)a	18 (22.8 ± 2.6)a	30 (39.6 ± 3.8)a	32.3 ± 2.6
Ia-1 µM	180	83 (46.1 ± 6.4)	12 (15.9 ± 4.6)a	11 (11.7 ± 3.1)a	8 (8.7 ± 3.6)ab	29 (36.8 ± 6.9)b	23 (26.8 ± 5.0)b	33.2 ± 2.4
Ia-2.5 µM	180	97 (53.9 ± 3.8)	33 (34.1 ± 4.8)b	37 (37.5 ± 5.3)b	20 (21.4 ± 5.2)a	7 (7.0 ± 2.9)c	0c	-

*Six replicated trials were carried out. Percentages of cleaved embryos are based on number of examined zygotes and are expressed as mean ± SEM. Cell numbers are expressed as mean ± SEM. **All embryos were classified after fixation and staining. Percentages of developmental stages are expressed as mean of total number of cleaved embryos ± SEM. a,b,c Values with different letters within a column indicates significant differences, $P < 0.05$.

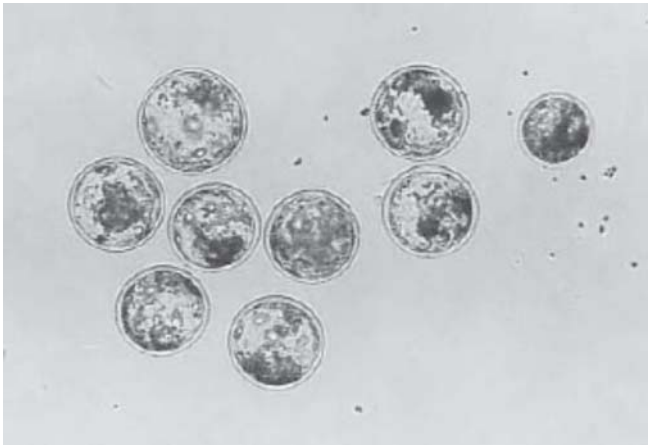

Figure 1. Porcine blastocysts were produced *in vitro* from IVM/IVF oocytes at day 6 after insemination (original magnification: x100).

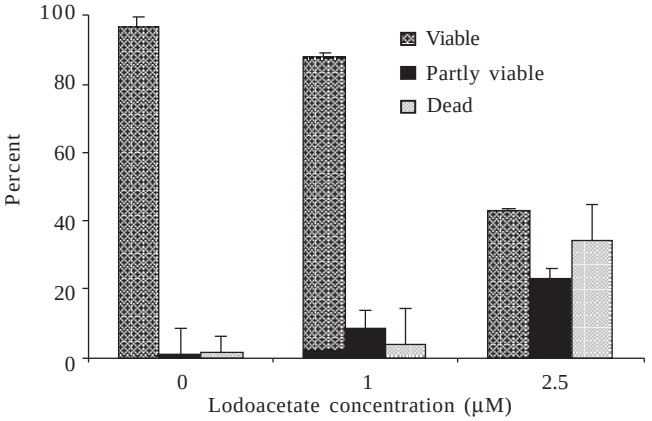

Figure 2. Viability of day 4 porcine embryos after culturing in medium supplemented with iodoacetate (Ia) for the first 2 days culture. Cleaved embryos (n= 79, 79, and 82 for Control, Ia-1 and Ia-2.5 group respectively) were collected at day 4 of culture, and then stained with FDA. Five replicated trials were carried out. Bars indicate error standard.

were arrested at the two-four-cell stage, and the embryos that could past the 4-cell stage to the further developmental stage were arrested at the morula stage (7%).

When the embryos were incubated with FDA for 15 min and examined under fluorescence microscope, 97% of control embryos were viable (Figure 2). Although, the rate of viable embryo in Ia-1 group (88.1%) was similar with control embryos, partly viable embryos in this group (8.7%) tended to be higher than those embryos of control group (1.2%). The proportion of viable embryos decreased when Ia was added to the culture medium at concentration of 2.5 µM (42.8%) ($P < 0.05$). About 34 and 23% of the total cleaved embryos in this group were already dead and partly viable, respectively.

DISCUSSION

Embryo metabolism is an important indicator of embryo viability as well as the efficiency *in vitro* culture systems. Understanding the metabolic needs of preimplantation embryos is vital to optimize its growth. If the necessary energy substrates are not present in sufficient concentrations or at the appropriate time, the embryo will unable to develop. One recurring pattern observed in embryo metabolism is the increasing of glycolysis and glucose usage in preimplantation development (Flood & Wiebold 1988; Rieger *et al*. 1992). During preimplantation embryo development, glucose is used during different stages and via different pathways. Glucose can be stored as glycogen via the pentose phosphate pathway, or enter the glycolytic pathway. The selection of pathways depends on the activity of certain key enzymes (Leppens-Luisier & Sakkas 1997).

To test the involvement of glycolysis in early stage of porcine embryo development, our study was carried out to examine whether iodoacetate, a glycolytic inhibitor, inhibits the developmental ability of the porcine embryo. Previous study showed that the inhibition of GAPDH result in the accumulation of glycolytic intermediates upstream of the conversion of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate by GAPDH (Janero *et al*. 1994; Danshina *et al*. 2001). In addition, the inhibition of GAPDH lead to increase flux through the polyol and hexosamine pathways and has been suggested to lead to diabetic embryopathy (Du *et al*. 2000; Wentzel *et al*. 2003). Wentzel *et al*. (2003) demonstrated that the inhibition of GAPDH activity by iodoacetate in gestational day 11 rat embryos results in increased malformation rates as well as decreased size, somite number, and DNA and protein content. Whereas Riley *et al*. (2006) reported that impairment of glucose metabolism via iodoacetate, a glyceraldehydes-3-phosphate dehydrogenase inhibitor, is sufficient to induce cell death programe in the embryos and decrease the quality of the blastocyst.

In the present study, we demonstrated that when porcine embryos at early stage of development were cultured in medium containing glucose as a sole energy source, glycolysis pathway play a key role on the generation of energy for embryo survival. The inhibition of this pathway result in a reduction in the number of cleaved embryos developed to the blastocyst stage. After culturing of IVF zygote with iodoacetate, in the first 2 days, the proportion of cleaved embryos developed to the blastocysts was decreased with the increasing of Ia concentration. When the embryos were incubated with FDA, partly viable embryos in Ia-1 group tended to be higher than those of control group; most of these embryos arrest developed at morula stage. Moreover, the proportion of viable embryos decreased significantly at concentration 2.5 μM and more than 60% of these embryos were arrested at the two-four-cell stage. The embryos that could past the 4-cell stage to the further developmental stage were arrested at the morula stage. These data indicate that culturing of early developmental stages of porcine embryos with iodoacetate cause an in vitro developmental block at the two-four-cell stage. The two-cell-block phenomenon has been defined functionally as an inability of the embryos to develop *in vitro* past to the two-cell stage in media that nonetheless supported the development of the embryo up to the four-cell stage (Seshagiri & Bavister 1991; Barnet & Bavister 1996).

In other species such as mice (Leese & Barton 1984), cows (Rieger *et al*. 1992; Kim *et al*. 1993), sheep (Gardner *et al*. 1993), and humans (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 & Bavister 1988; Thompson *et al*. 1992; Kim *et al*. 1993). However, the results of the present study showed that inhibition of glucose metabolism may disrupt the management of energy production, leaving the embryos incompetence to develop. Therefore, the maintenance of glycolysis pathway at early stage of porcine embryo development is importance for the success of subsequent developmental. Our findings support a previous report of glucose metabolism by pig embryos (Gandhi *et al*. 2001; Swain *et al.* 2002).

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