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Developmental Competence of Early Stage Porcine Embryos Cultured in Medium with Different Energy Substrate *in vitro*

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Abstract

To elucidate the effect of energy requirement during the early embryonic development on their developmental ability to develop to blastocyst stage, in vitro fertilized (IVF) porcine one-cell embryos were cultured in modified North Carolina State University (NCSU)-37 supplemented with different energy substrate. Result indicated that the cleavage rate of embryos in Pyr-Lac and Gluc-Pyr-Lact groups was significantly higher than in those in Gluc group and Gluc-Rib group ($P < 0.05$). At Day 6 of culture, the highest proportion of embryos develop to the blastocyst stage was obtained in the presence of pyruvate-lactate only. In the medium with glucose, the addition of pyruvate-lactate or ribose slightly increased the proportion of embryos develop to the blastocyst stage, however the value were not significantly different form those obtained in the presence of glucose only. The mean cell number in blastocysts derived from Pyr-Lac and Gluc-Pyr-Lact groups were significantly higher than those in the Gluc group ($P < 0.05$). These results indicated that the presence of glucose only, as energy substrate, during the first 2 days of in vitro culture (IVC) caused a decrease in development of in vitro produced (IVP) porcine embryos to the blastocyst stage and mean cell number in blastocysts .

Keywords: porcine embryos-energy substrate-in vitro culture

Introduction

The development of culture system to support early embryonic development is important for study of the factor that are involved as well as for practical use in such biotechnologies as in vitro fertilization (IVF), nuclear transplantation and gene transfer by DNA microinjection. Embryos from most species exhibit less than optimal development in vitro and may 'block' during development at specific cell stages (Bavister, 1979). Thus, the culture medium or environmental conditions used for *in vitro* culture must be inferior for supporting normal development compare than *in vivo* condition. *In vitro* culture conditions deviate from *in vivo* situations in many respects, but one of the critical factors appears to be the energy substrates present in culture medium for the production of energy and for the synthesis of a variety of complex molecules, such us glucose, pyruvate and lactate (Rieger *et al.*, 1992).

It is well known that metabolic activity and substrates preferences of embryos appear to change between early and late

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cleavages with elevated glucose and oxygen consumption as they approach cavitation (Houghton *et al.*, 1996). Most mammalian embryos need pyruvate as a substrate of energy in the early stages of development (Rieger *et al.*, 1992; Gardner *et al.*, 1993). Pyruvate metabolism was relatively high at the two- and four-cell stages, declined to a minimum at the compacted morula stage, and then increased at blastulation. Glucose metabolism via glycolysis, on the other hand, increased between the eight-cell and 16-cell stage, and continued to increase up to the blastocyst stage (Rieger *et al.*, 1992). Similarly, glucose uptake by sheep embryos is low before the 16-cell stage and increases steadily thereafter (Gardner *et al.*, 1993). Unlike cattle embryos, pyruvate uptake by sheep embryos is relatively constant up to the morula stage and then increases significantly through the blastocyst stage (Thompson *et al.*, 1992; Gardner *et al.*, 1993). In the mouse, although the zygote and early cleavage stage embryo cannot use glucose as the sole substrate of energy, the blastocysts exhibit a large capacity to utilize glucose both oxidatively and through aerobic glycolysis (Chatot *et al.*, *1989*). In pigs, it has been reported that the embryos consumed glucose and produced lactate at all stages of development, utilization of glucose was low during the early cleavage stages, increased slightly at the morula stage and reached a peak at the early blastocyst stage (Sturmey and Leese, 2003). Therefore, the uptake and metabolism of energy substrate such as, glucose and pyruvate are appears to be developmentally related.

Although IVF and embryo production using *in vitro* matured oocytes have been successful and many laboratories have tried to overcome inadequacies of their IVC system by supplementing culture medium with various energy substrate or using stepwise culture system with components that may be advantageous for different stages of embryo development (Petters and Wells 1993; Gandhi *et al.*, 2001; Kikuchi *et al.*, 2002; Karja *et al.*, 2004a, 2004b), their developmental ability and the quality of blastocyst yielded are also still low compared to in vivo-derived blastocyst. Therefore, the purpose of this study was to observe developmental ability of early stage porcine embryos after culturing in medium with different energy substrate. We have focused specifically the experiment on the first 2 days of culture, early stage of embryo development, since the first 2 days of culture in porcine embryos seem to have a critical effect on further development (Kikuchi *et al.*, 2002), at period when the activation of embryonic genome occurs. In the suboptimal *in vitro* culture conditions, embryos are blocked in developmental or slowing down in cleavages at the stage when the activation of embryonic genome occurs (Telford *et al.*, 1990)

Materials and Methods

Oocyte collection and in vitro maturation (IVM)

Collection and IVM of porcine follicular oocytes were performed according to Kikuchi *et al.* (2002). Briefly, ovaries from prepubertal cross-bred gilts (Landrace X Large White) were collected at a local slaughterhouse and carried to the laboratory in Dulbecco's PBS (Nissui Pharmaceutical Co. Ltd) at 35–37 8C within 1 h. Cumulus–oocyte complexes (COCs) were collected by scraping of 3–6 mm follicles in a collection medium consisting of Medium 199 (with Hanks' salts; Sigma Chemical Co.) supplemented with 10% fetal bovine serum (Gibco and Invitrogen Corp.), 20 mM HEPES (Dojindo Laboratories), and antibiotics (100 units/ml penicillin G potassium (Sigma) and 0.1 mg/ml streptomycin sulfate (Sigma)). Maturation culture was performed in a modified North Carolina State University (NCSU)-37 solution (Petters and

Wells, 1993) containing 10% (v/v) porcine follicular fluid, 0.6 mM cysteine, 1 mM dibutyryl cAMP (dbcAMP, Sigma), 10 IU/ml eCG (PMS 1000 Tani NZ, Nihon Zenyaku Kogyo), and 10 IU/ml hCG (Puberogen, 500 U, Sankyo) in a four-well dishes (Nunclon Multidishes) for 22 h in an atmosphere of 5% CO2, 5%O2, and 90% N2 at 39°C. COCs were subsequently cultured in maturation medium without dbcAMP and hormones for an additional 24 h under the same atmosphere.

In vitro fertilization (IVF)

Spermatozoa were thawed and preincubated for 15 min at 38.5°C in a tissue culture medium (TCM) 199 with Earle's salts (Gibco) 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. Fertilization medium consists of 90 mM NaCl, 12 mM KCl, 25 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 0.5 mM $MgSO_4$, 10 mM sodium lactate, 3 mg/ml bovine serum albumin (BSA; Fatty acid free, Sigma), 5 mM caffeine (Sigma) and 50 µg/ml gentamicin. The final sperm concentration was adjusted to 1 x 10^6/ml. The oocytes were co-incubated with spermatozoa for 3 h. And then, the inseminated oocytes were denuded from the cumulus cells and attached spermatozoa by mechanical pipetting and transferred to culture medium to which they had been designed, as bellow.

Embryo culture

The basic medium used for embryo culture was glucose-free NCSU-37 containing 4 mg/ml BSA and 50µM b-mercaptoethanol (IVC medium). To observe developmental ability o developmental ability of early stage porcine embryos after culturing in medium with different energy substrate for the first 2 days of culture, presumptive zygotes were cultured from Days 0 (the day of insemination was defined as day 0) to 2 in IVC medium supplemented with (1) 5.5 mM glucose (Gluc group), (2) 0.17 mM sodium pyruvate and 2.73 mM lactate (Pyr-Lact group), (3) 5.5 mM glucose, 0.17 mM pyruvate and 2.73 mM lactate (Gluc-Pyr-Lact group), or (4) 5.5 mM glucose and 5.5 mM Ribose-5 phosphate (Gluc-Rib group). Subsequently, embryos in all groups were cultured until Day 6 in IVC medium supplemented with 5.5 mM glucose only. Culture was performed at 38.5°C and 5% CO_2 under 5% O_2 and 90% N_2.

Embryos evaluation

For examination of the embryo development, on Day 6 all embryos in all groups 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 0.3ÿ (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.). A coverslip was placed on top of the embryos and the staining of the nuclei was examined with a fluorescence microscope (BX 51; Olympus). The numbers of cleaved embryos including the stage of embryo development, blastocysts formation rate, and the cell number in blastocyst were recorded.

Statistical Analysis

All percentage data were subjected to arc-sine transformation before statistical analysis. Then all data, including the arcsine-transformed percentages of blastocyst formation and the data on mean number of cells in the blastocyst were subjected to ANOVA by a post hoc, Fisher's protected least significant difference test

(PLSD test) using the STATVIEW (Abacus Concepts Inc., Berkeley, CA, USA) program. Data were expressed as means ± SEM. Differences at P < 0.05 were considered significant.

Results and Discussion

The results of *in vitro* cleavage and development of porcine zygotes to the blastocyst stage and total cell number in blastocyst are shown in Table 1. When the embryos were cultured in the medium without any energy substrate, no cleavage and development to blastocyst stage were observed. In the medium supplemented with pyruvate-lactate only (Pyr-Lac group) or together with glucose (Gluc-Pyr-Lact groups), the proportion of embryos that cleaved at 48 hours after IVC was significantly higher than in medium with glucose only (Gluc group) or together with ribose (Gluc-Rib group) ($P < 0.05$). At Day 6 of IVC, the highest proportion of embryos develop to the blastocyst stage was obtained in the presence of pyruvate-lactate only. In the medium with glucose, the addition of pyruvate and lactate or ribose slightly increased the proportion of embryos develop to blastocyst stage, but the values were not significantly different from those obtained in gluc-group.

Table 1. Developmental competence of porcine embryos cultured in medium with different energy substrates during early embryonic development

Groups	No. of oocytes number of	Mean ± SEM (No.) of cleaved examined	Mean± SEM (No.) of embryos embryos to blastocyst	Mean ± SEM of total cell developed blastocyst
Gluc	120	40.8 ± 2.8 (49)[a]	13.3 ± 1.4 (16)[a]	32.6 ± 2.7[a]
Pyr-lact	115	68.0 ± 4.9 (80)[b]	24.2 ± 3.1 (29)[b]	40.8 ± 2.8[b]
Gluc-Pyr-Lact	147	64.7 ± 4.2 (97)[b]	19.7 ± 3.4 (23)[ab]	40.7 ± 2.9[b]
Gluc-Rib	110	46.7 ± 7.8 (52)[b]	21.3 ± 4.7 (24)[ab]	35.5 ± 2.2[ab]
None	125	0	0	

Four replicate trials were carried out.
Values are expressed as means ± SEM
[a-b] values with different superscript letters are significantly different ($p<0.05$)

As shown in Table 1, the mean cell number in blastocysts derived from Pyr-Lac and Gluc-Pyr-Lact groups were significantly higher than those in the Gluc group ($P < 0.05$). Developed porcine embryos at blastocyst stage and blastocyst with Hoechst-labeled nuclei at Day 6 of culture were represented in Figure 1.

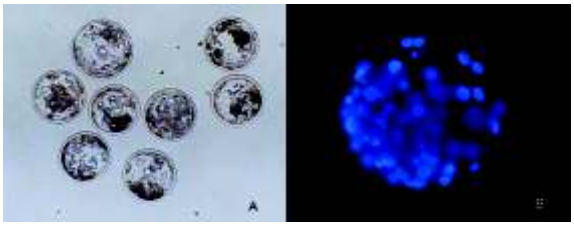

Figure 1. Developed porcine embryos at blastocyst stage (A) and blastocyst with Hoechst-labeled nuclei (B) at Day 6 of culture (original magnification 100x).

The types and concentrations of energy substrates added to a culture medium alter metabolic profiles, development and quality of embryos. The results from this study has demonstrated that when porcine IVM-IVF oocytes were cultured in IVC medium supplemented with pyruvate and lactate for the first 2 days and then cultured in the medium containing glucose for subsequent 4 days, the rate of blastocyst formation the cell number in blastocysts were higher as compared with that of glucose supplement for the first 2 days. Although porcine embryos has been reported are able to utilize glucose throughout their development, results from the present study indicate that glucose alone as energy substrate during early stage of porcine embryos development could not support development of the embryos to the blastocyst stage. The lack of response to the glucose in this study could be explained as the limited ability of early stage

porcine embryos to utilize glucose (Sturmey and Leese, 2003) or the embryos use only a fraction of the glucose that they take up (Swain *et al.,* 2002). Porcine embryos appear to behave similar fashion metabolically as do other domestic livestock species embryos, with limited glucose utilization before the morula stage and a heavy reliance on glycolysis during blastocoele formation (Flood and Wiebold, 1988; Rieger *et al.*, 1992; Gandhi *et al.*, 2001). On the other hand, culturing porcine embryos in a combined medium that provided 5.5 mM glucose, pyruvate and lactate, or glucose combining with 5.5 mM ribose 5-phosphate, the end product of glucose metabolism through pentose phosphate pathway (PPP), as energy substrates for the first 2 days and followed by culture in medium with glucose (5.5 mM) the next 4 days slightly improved blastocyst development and cell number in blastocysts compared with those cultured for 144 h in medium with glucose. Taken together, these results clearly indicate that glucose in IVC medium for the first 2 days of culture is detrimental to the development of embryos, while pyruvate and lactate appear to be important energy substrates for both early embryonic development in vitro and for improving the number of cells in blatocysts. Our observation confirms the previous reports, which reported that pyruvate and lactate sseem to be predominant energy substrates for the first cleavage division for porcine embryos (Kikuchi, 2002, Kim *et al.*, 2004). Pyruvate and lactate may also provide the embryos with more suitable conditions for cellular oxidation reduction equilibrium, resulting in viable embryo growth and development (Thompson *et al.* 1993). Butcher *et al.* (1998) recently suggested that pyruvate might be converted to alanine and may play a role in removing ammonia from embryos. Another possible function for pyruvate is to protect embryos against oxidative stress as suggested by Leese (1991).

Glucose is usually included in standard tissue culture media at a concentration of 5.5 mM, on the basis of its concentration in serum. This is much higher compared than in the oviduct, in which in vivo porcine embryos are exposed (0.17 mmol glucose l^{-1}) (Nichol *et al.*, 1992). The deleterious effects of glucose on embryo development in vitro have been reported. The most likely reason that glucose affects the development of early stage embryo, therefore, is that one or more metabolites generated by the PPP leads to a failure in development of the embryos. One possibility is that NADPH, although generally regarded as protective against free radical damage (Salvemini *et al.,* 1999), is produced in such quantities that its re-oxidation actually generates oxygen free radicals, an occurrence that is well documented in some cell types (Babior, 2000). The use of glucose as an energy substrate results in the production of reactive oxygen species (ROS), particularly the superoxide anion and the hydroxyl radical. Production of superoxide anion and H_2O_2 via glucose metabolism through PPP has been described on rabbit blastocyst (Manes and Lai, 1995). Reactive oxygen species are highly active electron acceptors, able to strip electrons from other molecules that, in turn, become free radicals. Hydrogen peroxide (H_2O_2) is not a radical per se, but is a product of O_2É ?and metal ion catalysis. However, both H_2O_2 and O_2É can form the extremely reactive OHÉ. The over-generation of intracellular ROS during culture of mammalian embryos in vitro is generally thought to be detrimental to embryo development (Johnson and Nasr-Esfahani, 1994; Guerin *et al.*, 2001).

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