Effect of Glucose and Sodium Phosphate on *In Vitro* Development of Porcine Embryos

Lee, S. H., S. M. Lim, S. Y. Lee[1], H. T. Cheong, B. K. Yang and C. K. Park[†]

College of Animal Resources Sciences, Kangwon National University, Chunchon, 200-701, Korea

ABSTRACT

This study was carried out to evaluate the effects of glucose and sodium phosphate on *in vitro* development of porcine oocytes matured and fertilized *in vitro*. When the culture medium was supplemented with various concentrations of glucose, the higher proportions (23 and 26%) of oocytes developed to morular or blastocyst stages were at the concentrations of 2.78 and 5.56 mM than 0 (9%; $P<0.05$) and 11.12 mM (18%). In experiment to evaluate effect of sodium phosphate during *in vitro* development of porcine oocytes, a significantly ($P<0.05$) higher proportions of embryos developed to morular or blastocyst stages was obtained with sodium phosphateof 0.28 (25%) and 0.53 (27%) mM than 0 (15%), 1.05 (19%) and 2.10 (10%) mM. On the other hand, when oocytes were cultured in medium with (0.53 mM) sodium phosphate, the proportions of developed embryos were significantly ($P<0.05$) higher in medium without (29%) that than with (14%) 5.56 mM glucose. However, a higher proportion of embryos developed to morular or blastocyst stages were obtained in medium with (23%) that than without (8%) glucose ($P<0.05$). The minimum essential medium (MEM) added to the culture medium were higher regardless of presence of sodium phosphate and glucose on the development of embryos. Although sodium phosphate and glucose could support morular and blastocyst development to a limited extend (10~24%), significantly higher proportion (36%) at morular or blastocyst stages was obtained by MEM adding in the medium with sodium phosphate and glucose. These results suggest that the early development of *in vitro* fertilized porcine oocytes can be maintained efficiently by glucose and sodium phosphate when they were cultured in medium with MEM.

(Key words : Glucose, *In vitro* development, Porcine embryos, Sodium phosphate, Minimum essential medium)

INTRODUCTION

Maintaining viability and development of early embryos during culture is crucial to a number of applications in the field of animal biotechnology. In the routine culture of preimplantationembryos, a single culture medium is used, leading to a static environment that does not allow for the metabolic and developmental changes that the embryo experiences during normal development. Efforts to culture different embryos met various successes, porcine embryos were regarded to be extremely difficult to culture past the 4-cell stage (Machaty et al., 1998).

Early studies (Brinster, 1965) showed that glucose cannot support the early stages of mouse embryo development. There is also considerable new evidence that inclusion of glucose in the culture medium at levels similar to those in plasma is inhibitory role during the early stages in many species, including the hamster (Barnett and Bavister, 1996), rat (Miyoshi et al., 1994), cow (Kim et al., 1993), sheep (Thompson et al., 1992) and human (Quinn, 1995). Because the mode in which glucose is inhibitory to production of energy by the embryo is not completely understood, it is plausible that other energy substrates may be more suitable in providing the embryo with its energy needs, while not inhibiting early development. On the other hand, the combination of glucose and phosphate arrests development (Seshagiri and Bavister, 1989), reduces respiration (Seshagiri and Bavister, 1991), and disrupts mitochondrial organization (Barnett et al., 1997) in the hamster.

Although the achievements made early stages of

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[1] Advanced Swine Research Institute

[†] Corresponding author : Division of Animal Resources Sciences, Kangwon National University, Chunchon, 200-701, Korea. E-mail : parkck@kangwon.ac.kr

embryonic development feasible *in vitro*, the adequate culture conditions for the preimplantation porcine embryo have yet to be determined. In the simpledefind medium, glucose or phosphate alone had no effect on the development of bovine embryos, but glucose together with phosphate inhibited embryo development to the blastocyst stage in comparison to the treatment with phosphate alone (Pinyopummintr and Bavister, 1991). Most of the previous research involving has been performed in the presence of phosphate, and phosphate may be responsible for developmental inhibition previously attributed to both glucose and phosphate.

The objective of the present study was to define culture conditions that provide the best *in vitro* environment for development of early porcine embryos: 1) different concentrations of glucose and sodium phosphate in a medium were evaluated, and 2) effect of minimun essential medium in medium combined with glucose and/or sodium phosphate were tested.

MATERIALS AND METHODS

Oocytes Preparation

Porcine ovaries were collected from a local slaughterhouse and kept in saline (NaCl, 0.9% w/v ; penicillin (100,000 IU/L) ; streptomycin (100 mg/L) ; and amphotericin B (250μg/L; Sigma Chemical, St-Louis, MO, USA) at 32 to 37℃. Cumulus-oocytes complexes were aspirated from 1- to 5-mm follicles with a 10-ml syringe with an 18-G needle. Oocytes were washed 3 times in Hepes-buffered Tyrode's medium (TLH) and once in maturation medium. Oocytes with a compact and complete cumulus were introduced into droplets of maturation medium (10 oocytes/50-$\mu\ell$ droplet), covered with mineral oil and cultured under an atmosphere of 5% CO_2 in air at 39℃ for 42~44 h. The maturation medium consisted of TC-199 medium with Earle's salt (Gibco, Lab., NY, USA) supplemented with 3.05 mM glucose, 0.32 mM Ca-lactate, 2.5 mM Hepes (Sigma), 10% fetal calf serum (FCS), 0.2 mM Na-pyruvate (Sigma), 50 μg/ml gentamycin (Sigma), 1 μg/ml FSH (sigma), 5 μg/ml LH (Sigma), 1 μg/ml estradiol 17β (Sigma) and 10% (v/v) porcine follicular fluid. During the culture, the hormones were removed from maturation medium for a second period of 21~22 h.

Semen Collection and Preparation

Semen from the sperm rich fraction of the ejaculate was collected from 3 boars that were housed at the National Livestock Research Institute. The semen was processed according to the straw freezing procedure described by Soejima et al. (1983) and Almlid and Johnson (1988) as modified by Lee et al. (1997). Briefly, the semen was transported to the laboratory at room temperature (approximately 24℃) and diluted 1:1 with BTS within 30 minutes after collection after evaluating the concentration and motility. Aliquots containing 6×10^9 spermatozoa were centrifuged for 10 min at 1,500 rpm. The seminal plasma was removed and the spermatozoa resuspended to 5 ml with the 1st cryodiluent. The diluted semen was cooled gradually to 4℃ for 1~2 h by placing the tubes in a 50 ml conical tube containing 30 ml water (room temperature); 5 ml ofthe 2nd cryodiluent containing cryoprotectants were added gradually for 1 h. The diluted semen samples equilibrated for 40 min at 4 ℃ and was packed into straws before being pre-frozen horizontally for 20 min on a thick paper rack, 5 cm (approximately -120℃) above LN_2 vapor in a styrofoam container (Almlid and Johnson, 1988). The pre-frozen semen was finally transferred directly into LN_2 for storage.

In Vitro Fertilization

The spermatozoa treatment for IVF used the method described by Park and Sirard (1996). Briefly, frozen straw was thawed by immersion in a water bath at 37℃ for 30 sec. The thawed semen was diluted with 2 ml of BTS (Beltsville Thawing Solution) and equilibrated in air-tight tubes at 37℃ in a waterbath for 10 min. After equilibration, the 2 ml of semen was separated over 2 layers of Percoll (65 and 70%) and centrifuged at 2000×G for 15 min at 20℃. The spermatozoa in the 65% Percoll layer were carefully collected, washed in the preincubation medium by suspension and re-centrifugation twice at 250×G for 10 min and resuspended in preincubation medium.

The concentration of motile spermatozoa after the final wash was adjusted to 25×10^6 cells/ml. The fertilization medium was TCM-199 supplemented with 3 mM glucose, 3 mM Ca-lactate, 0.2 mM Na-pyruvate and 10% FCS. The final concentration of spermatozoa was adjusted to 1×10^6 cells/ml motile sperm cells during fertilization.

Embryo Culture

In-vitro fertilized oocytes were cultured in different conditions. The basic medium used for culture was NCSU-23 medium supplemented with 4 mg/ml BSA. In all experiments the oocytes cultured at 39℃ in atmosphere of 5% CO_2-95% air with high humidity.

In Experiment 1, to examine the effect of concentration of glucose on embryo development, oocytes at 8 h after insemination were transferred into medium with 0, 2.78, 5.56 and 11.12 mM of glucose and then cultured further for 144 h.

In Experiment 2, oocytes were freed from cumulus cells at 8 h post-insemination through a fine pipette in medium with 0.1% hyaluronidase and washed four times

with culture medium. 10 oocytes were transferred into 0.5ml NCSU-23 medium with 0, 0.28, 0.53, 1.05 and 2.10 mM of sodium phosphate and then cultured for 144 h.

In Experiment 3, the combined effects of glucose (5.56 mM) and sodium phosphate (0.53 mM) on the development of embryos were examined in a 2× 2 factorial design each in the absence and presence of glucose or sodium phosphate.

In Experiment 4, to examine whether the addition of minimum essential medium (20 $\mu\ell$/ml) during the culture of porcine embryos, they were examined the effect of minium essential medium in NCSU-23 medium with glucose and/or sodium phosphate. Medium was changed every two days during the culture in all experiments.

Statistical Analysis

Data were analyzed by Student's *t*-test using GLM procedures and Duncan's multiple range test.

RESULTS

In Experiment 1, when oocytes had been cultured with different concentrations of glucose, there were more developed to early embryo in medium with that than without glucose (Fig. 1). The proportions of embryos developed to morular and blastocyst stages were higher ($P<0.05$) in medium containing 2.78 and 5.56 mM than 0 and 11.12 mM glucose. The highest proportion (26%) was obtained from medium with 5.56 mM glucose.

In Experiment 2, when the culture mediumwas supplemented with various concentrations of sodium phosphate (Fig. 2), the higher proportions (25 and 27%) of oocytes developed to morular or blastocyst stages were obtained at the concentrations of 0.28 and 0.53 mM than 0(15%), 1.05(19%) and 2.10(10%) mM sodium phosphate ($P<0.05$).

In Experiment 3, theoocytes were cultured in medium with (0.53 mM) sodium phosphate. The proportions of developed embryos were significantly ($P<0.05$) higher in medium without (29%) that than with (14%) 5.56 mM glucose (Fig. 3). However, a higher proportion of embryos developed to morular or blastocyst stages were obtained in medium with (23%) that than without (8%) glucose ($P<0.05$) in medium without sodium phosphate.

In Experiment 4, The minimum essential medium (MEM) added to the culture medium were higher regardless of presence of sodium phosphate and glucose on the development of embryos (Table 1). Although sodium phosphate and glucose could support morular and blastocyst development to a limited extend (10~24%), significantly higher proportion (36%) in morular or blastocyst stages was obtained by MEM adding in the medium with sodium phosphate and glucose.

DISCUSSION

Glucose has been related to several aspects of oocyte and sperm physiology. There has been considerable debate surrounding the inclusion of glucose in embryo culture media. Several studies have shown that high concentrations of glucose are detrimental to embryo development in culture (Thompson et al., 1992; Quinn, 1995). Energy substrate such as glucose is present in most culture media used for *in vitro* culture of oocytes and is a major precusor of DNA synthesis and phospholipid synthesis (Stryer, 1995). Glucose is certainly found in the female reproductive tract (Gardner and Leese, 1990; Nichol et al., 1992) and is preferred energy substrate after compaction for many species of mammalian embryos, implying a physiological role for glucose in culture.

The present study indicate that porcine oocytes matured and fertilized *in vitro* can developed to the morular and blastocysts stages in medium with 2.78 and 5.56 mM glucose. High concentration (11.12 mM) of glucose, however, was detrimental to embryo development. These results should not be surprising, because concentrations of glucose in the female reproductive tract are considerably lower than those in plasma and drop additionally following ovulation (Gardner et al., 1996). Ludwig et al. (2001) reported that although no inhibition was found, low concentrations of glucose have little benefit for preimplantation hamster embryo development in culture. They also demonstrated that preimplantation embryo exposure to hexoses is not necessary to obtain adequate numbers of embryos developing to the blastocyst stage but is important for the continuing developmental competence of the blastocyst and its ability to undergo successful implantation.

The sodium phosphate (0.28 and 0.53 mM) has beneficial effects on porcine embryo development in NCSU-23 medium. The result clearly indicates that the presence of an adequate concentration (0.53 mM) of sodium phosphate in culture medium without glucose is essential for the development to morula and blastocyst stages (Fig. 3). It is reported in almost the same condition that the presence of sodium phosphate alone have no effect on bovine embryo development to the blastocyst stage (Pinyopummintr and Bavister, 1991). However, there have been no comparable reports about the effects of sodium phosphate on development of porcine embryos in the chemically defined condition. The requirement of sodium phosphate in porcine embryo development is not different from bovine embryos in which developments to the morula and blastocyst stages (Kim et al., 1993).

In this study, adding of 20 $\mu\ell$/ml minimum essential medium(MEM) to culture medium enhanced porcine embryo development in medium with that than without

glucose. Studies on the effect of amino acid supplementation on the *in vitro* development of embryos of livestock species have concentrated on the evaluation of media containing amino acids at concentrations used by Eagle (1959). Generally, supplementation has improved embryo development in the rabbit (Kane and Foote, 1970), hamsters (Bavister et al., 1983), rats (Zhang and Armstrong, 1990), porcine (Rosenkrans et al., 1989) and bovine (Takahashi and First, 1992). In our study, improvements in embryo morphology and rates of blastocyst development were obtained by MEM containing glucose and sodium phosphate. It is possible that membranes of embryos cultured in glucose-free media containing sodium phosphate are sufficiently stable to negate any long-term benefit including glucose in the medium. In conclusion, this study has demonstrated that IVM/IVF porcine zygotes cultured in NCSU-23medium with glucose, sodium phosphate and MEM are able to develop into morula and blastocyst stages at relatively high rates.

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