Effect of Osmolarity of Culture Medium on the Preimplantation Development of Porcine NT and IVF Embryos

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ABSTRACT

In vitro **development of porcine embryo is affected by culture condition. One possible factor is osmolarity of culture medium. This study examined whether high osmolarity of culture medium at the early culture stage improves development of preimplantation porcine *in vitro* fertilization (IVF) and nuclear transfer (NT) embryos. NT and IVF embryos were divided into three groups and the basic medium was PZM-3 (250~270 mOsmol, control group). The control group of embryos was cultured in PZM-3 for whole culture period. Other two groups of embryos were cultured in a modified PZM-3 with 0.05 M sorbitol or 0.05 M sucrose (300~320 mOsmol, sorbitol or sucrose group) for the first 2 days, and then cultured in PZM-3 for further culture. NT embryos cultured in sucrose group showed a significantly higher developmental rate to the blastocyst stage with a decreased apoptosis rate compared to the sorbitol ($p<0.05$). For IVF, sucrose group showed a significantly increased the blastocyst formation rate with a decreased apoptosis rate compared to the control ($p<0.05$). This study represents that the high osmolarity in the early embryo culture stage can enhance the *in vitro* development of porcine NT and IVF embryos to the blastocyst stage with reduced apoptosis of cells.**

(Key words : Osmolarity, NT embryos, Sorbitol, Sucrose, Apoptosis)

INTRODUCTION

Recent progress in the *in vitro* maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* development (IVD) in pig oocytes has made it possible to produce IVF-derived (Abeydeera *et al.*, 1998a) and somatic cell cloned piglets (Betthauser *et al.*, 2000; Onishi *et al.*, 2000). However, developmental rate to the blastocyst stage of porcine nuclear transfer (NT) embryos is still low. Also the efficiency of pig cloning through somatic cell NT has been extremely low with less than 1~5% of transferred embryos surviving to term (Walker *et al.*, 2002).

Studies have been performed to improve the developmental competence of porcine *in vitro* produced embryos (Abeydeera *et al.*, 1998b, 2000; Kano *et al.*, 1998; Andrew *et al.*, 2000; Wang *et al.*, 2000; Gandhi *et al.*, 2001) and have demonstrated that many factors are involved embryo development and viability after transfer. These include growth factors, oxygen, energy substrates, amino acids, and albumin (Petters *et al.*, 1990; Mercola *et al.*, 1998). Therefore, it is expected that components in culture medium are one of the important factors affecting embryo viability of porcine NT and IVF embryos. The *in vitro* culture system is one of the most useful strategies for understanding the mechanism of early embryonic development and establishing the optimum culture condition.

Osmolarity of culture media is one of the important factors affecting *in vitro* development of preimplantation mammalian embryos to the blastocyst stage. It was reported that the presence of organic osmolytes such as sorbitol in the maturation medium improved the microfilament organization at the end of *in vitro* maturation and during early development following IVF of porcine oocytes (Funahashi *et al.*, 1996). Recently, Im *et al.* (2005) reported that the addition of sorbitol and sucrose could reduce fragmentation and support better development to the blastocyst stage.

One of the serious problems for pig embryos produced *in vitro* is apoptosis. Programmed cell death or apoptosis is crucially involved in development and differentiation. Environmental stresses, such as those caused by *in vitro* culture, induce unscheduled apoptosis in cultured embryos, which may lead arrest or abnormal development and lower viability of embryos (Hardy *et al.*, 1989; Jurisicova *et al.*, 1998; Byrne *et al.*, 1999).

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Therefore, the present study was conducted to examine the effect of osmolarity of culture medium at the early embryo culture stage on the pre-implantation development of porcine NT and IVF embryos.

MATERIALS AND METHODS

Collection of Oocytes and IVM

Porcine ovaries were obtained from a local slaughterhouse and transported to the laboratory at 30~35℃. Cumulus-oocyte complexes (COCs) were collected by the aspiration of ovary antral follicles (3~6 mm diameter) with 18 gauge needle fixed to a 10 ml disposable syringe. The COCs with several layers of cumulus cells were selected and washed three times in maturation medium. For maturation culture, approximately 50~100 COCs were transferred into 500 μl of maturation medium (TCM-199, Gibco-BRL, Grand Island, NY, USA) covered with mineral oil in a four-well dish (Nunc, Roskilde, Denmark). Oocytes were matured for 40 to 44 hr at 38.5℃ under 5% CO_2 in air. The maturation medium supplemented with 0.1% PVA (w/v), 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 0.57 mM cysteine, 0.5 μg/ml lutenizing hormone, 0.5 μg/ ml follicle stimulating hormone, 75 μg/ml penicillin G and 50 μg/ml streptomycin.

IVF

Cumulus-free oocytes were washed three times in IVF medium. Approximately, 35~40 oocytes were transferred into 50 μl droplet of IVF medium covered with mineral oil that had been equilibrated for 40 hr at 38.5 ℃ in 5% CO_2 in air. The fertilization medium was a modified tris-buffered medium consisting of 113 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2 \cdot 2H_2O$, 5 mM sodium pyruvate, 11 mM glucose, 20 mM Tris, 2 mM caffeine, and 2 mg/ml of BSA. For IVF, fresh semen was collected from different boars at A.I center. Semen were diluted and equilibrated in air-tight tubes at 16℃. After equilibration, the 2 ml of semen were centrifuged twice at 1,200 rpm for 5 min and resuspended in fertilization medium. After the final wash, the concentration of motile sperm was adjusted to 5×10^5 cells/ml in fertilization medium. At 6 hr after insemination, oocytes were washed three times and cultured in 0.5 ml of different culture media in four-well dishes at 38.5℃ in 5% CO_2 in air.

Preparation of Porcine Fetal Fibroblast Cells

A day 35 porcine fetus was retrieved from pregnant gilt. After the brain, intestines, and four limbs were removed, tissue was cut into small pieces with fine scissors. Cells were incubated for 30 min at 39℃ in PBS supplemented with 0.05% trypsin and 0.02 mM EDTA (all chemicals, unless noted otherwise, were from Sigma, St. Louis, MO), and the suspension was centrifuged at 1,200 rpm for 5 min. The cell pellet was resuspended and cultured in Dulbecco's Modified Eagle's medium (DMEM; Gibco-BRL) supplemented with 15% fetal bovine serum and 75 μg/ml antibiotics. The cells were passaged two times, and then frozen by using DMEM supplemented with 10% dimethylsulfoxide (DMSO). To be used as donor cells in NT, cells were thawed and cultured until they reached confluence. Before NT, cells were treated with 0.05% trypsin for single-cell isolation. Fibroblast cells were cultured and passaged (2 to 8 passages) and used as donor cells for NT.

Production of NT Embryos

After maturation, cumulus cells were removed from oocytes by vortexing the COCs in PBS supplemented with 0.1% PVA and 0.1% hyaluronidase for 4 min. Oocytes were enucleated by the aspiration of the first polar body and metaphase-II (MII) plate in a small amount of surrounding cytoplasm (about 20% of oocyte volume) with a glass pipette. All micromanipulation procedures were performed in TCM-199 supplemented with 3 mg/ml BSA and 5 μg/ml cytochalasin B (CB). A single cell with a smooth surface was transferred into the perivitelline space of an enucleated oocyte. Reconstructed oocytes were then placed between 0.2 mm diameter wire electrodes (1 mm apart) of a fusion chamber overlaid with 0.3 M mannitol solution supplemented with 0.1 mM $MgSO_4$, 1.0 mM $CaCl_2$, and 0.5 mM Hepes. For the fusion, two DC pulses (1 sec interval) of 1.2 kV/cm were applied for 30 μsec using a BTX Electro Cell Manipulator 200 (BTX, San Diago, CA, USA). After fusion treatment, the reconstructed oocytes were cultured in culture medium for 30 min and the fusion was determined. Embryos were washed and transferred into each culture medium covered with mineral oil in a four-well dish. Basic culture medium was PZM-3 (Im *et al.*, 2004).

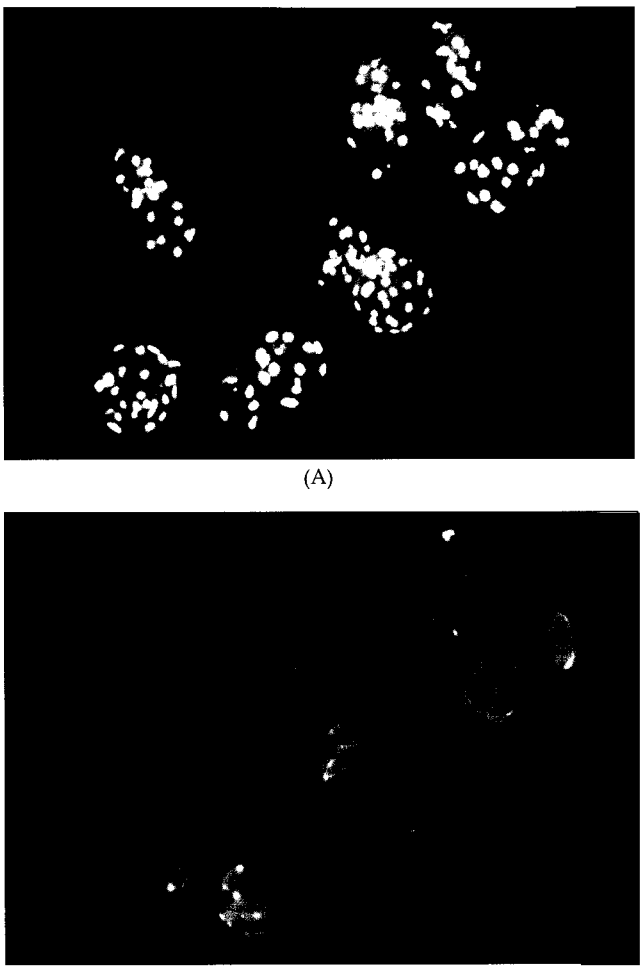
In Vitro Culture of Embryos (IVC)

Embryos were washed and transferred into each culture medium covered with mineral oil in a four-well dish. Basic culture medium was PZM-3 (Im *et al.*, 2004). On Days 2 and 6 of culture, the cleavage and development to the blastocyst stage were examined, respectively. After 6 days of *in vitro* culture, total cell numbers, and apoptosis of cells in all blastocysts were examined.

Apoptosis Assays

The blastocysts on Days 6 from NT and IVF were

washed twice in PBS/PVP (PBS supplemented with 0.1 % polyvinylpyrolidone) and fixed in 4% (v/v) paraformaldehyde solution for 24 hr at 4℃. Membranes were permeabilized in 0.5% Triton X-100 for 30 min at room temperature.



(A)

(B)

Fig. 1. Apoptosis in porcine NT blastocysts. A: total cells (light blue), B: apoptotic cells (red).

A TUNEL assay was used to assess the presence of apoptotic cells (*in situ* cell death detection kit, TMR red; Roche, Mannheim), for 1 hr at 38.5℃ in the dark. The broken DNA ends of the embryonic cells were labeled with TDT and fluorescein-dUTP. After the reaction stopped, the embryos were washed and transferred into 10 μg/ml Hoechest 33342 for 30 min at room temperature in the dark. The embryos were washed three times and mounted on slides with Prolong antifade Kit (cat. P-748, Molecular Probes, Eugene, OR, USA). The slides were stored at −20℃. The numbers of apoptotic nuclei and total numbers of nuclei were determined from optical images of whole-mount embryos (Fig. 1.) under an epifluorescent microscope (Nikon, Tokyo, Japan).

Statistical Analysis

To determine the statistical significance between treatments, the rates of cleavage and blastocyst formation, total cell number and percentage of apoptosis were subjected to a Generalized Linear Model procedure (PROC-GLM) of the Statistical Analysis System (SAS Institute, Cary, NC, USA). Differences among treatment means were determined by using the Duncan's multiple range-tests. All data were expressed as Least Square (LS) mean ± SEM (Standard Error of the sample Mean). Differences were considered significant at $p<0.05$.

RESULTS

Effects of Sorbitol and Sucrose on Development of NT Embryos

There was no difference between sorbitol and sucrose groups in cleavage rate, but developmental rate

Table 1. Effects of sorbitol and sucrose on the development of porcine NT embryos

Culture treatment*	No. of oocytes fused/ manipulated	No. of NT embryos cultured**	No. (%±SEM) of NT embryos developed to	
			≥ 2cell	Blastocyst
Control	375/475	125	89(71.2±15.1)	25(20.0±6.3)[ab]
Sorbitol		125	99(79.2± 8.6)	22(17.6±7.1)[b]
Sucrose		125	106(84.8± 2.8)	36(28.8±6.1)[a]

* Fused NT embryos were cultured in PZM-3 supplemented with 0.05 M sorbitol or sucrose for the first 2 days and then cultured in PZM-3 without sugars for further 4 days. For the control, NT embryos were cultured in PZM-3 without sugars for whole culture period.

** Fused oocytes were divided into three groups and cultured.

[a,b] Values with different superscripts differ significantly ($p<0.05$).

Table 2. Effects of sorbitol and sucrose on apoptosis in porcine NT blastocysts

Culture treatment*	No. of blastocysts	Total nuclei	Apoptotic cells	% TUNEL
Control	25	638	40	6.27±2.9[a]
Sorbitol	22	578	27	4.67±2.8[ab]
Sucrose	36	1,109	38	3.43±1.9[b]

* Fused NT embryos were cultured in PZM-3 supplemented with 0.05 M sorbitol or sucrose for the first 2 days and then cultured in PZM-3 without sugars for further 4 days. For the control, NT embryos were cultured in PZM-3 without sugars for whole culture period.

[a,b] Values with different superscripts differ significantly ($p<0.05$).

to the blastocyst stage was significantly higher ($p<0.05$) in the medium with 0.05 M sucrose group than 0.05 M sorbitol group (Table 1). Although blastocyst formation rate was not different from the control group, the apoptosis rate was significantly lower ($p<0.05$) in the medium with 0.05 M sucrose group than the control group (3.43±1.9% vs. 6.27±2.9%, respectively, Table 2).

Effects of Sorbitol and Sucrose on Development of IVF Embryos

There was no difference between sorbitol and sucrose groups in the cleavage and blastocyst rates, but developmental rate to the blastocyst stage was significantly higher ($p<0.05$) in the medium with 0.05 M sucrose group than the control group (32.3±6.4% vs. 17.9±2.7%, respectively, Table 3). Moreover, the apoptosis rate was significantly lower ($p<0.05$) in the medium with 0.05 M sucrose group than the control group (0.63±0.3% vs. 1.79±0.4%, respectively) (Table 4). Also, number of nuclei in the blastocysts was significantly higher ($p<0.05$) in the medium with 0.05 M sucrose group than the control group (Table 4).

Table 3. Effects of sorbitol and sucrose on the development of porcine IVF embryos

Culture treatment*	No. of oocytes cultured	No. (%±SEM) of embryos developed to	
		≥ 2cell	Blastocyst
Control	134	102(76.1±7.5)	24(17.9±2.7)[b]
Sorbitol	133	104(78.2±5.6)	33(24.8±2.5)[ab]
Sucrose	133	110(82.7±4.2)	43(32.3±6.4)[a]

* IVF embryos were cultured in PZM-3 supplemented with 0.05 M sorbitol or sucrose for the first 2 days and then cultured in PZM-3 without sugars for further 4 days. For the control, embryos were cultured in PZM-3 without sugars for whole culture period.

[a,b] Values with different superscripts differ significantly ($p<0.05$).

DISCUSSION

The present study shows that the osmolarity of culture medium plays an important role in *in vitro* development of porcine embryos. The results indicate that the development of porcine embryos in the early culture stage is largely dependant on the osmolarity of the medium. The optimal osmolarity of medium for development of rabbit 2-cell embryos has been reported as 230~339 mOsmol (Naglee *et al.*, 1969), and hamster 2- and 8-cell embryos show a wide range of optimal osmolarities, 250~325 mOsmol (Mckieran *et al.*, 1990) and 225~300 mOsmol (Bavister *et al.*, 1983), respec-

Table 4. Effects of sorbitol and sucrose on apoptosis in porcine IVF blastocysts

Culture treatment*	No. of blastocysts	Total nuclei	Apoptotic cells	% TUNEL
Control	24	839[b]	15[ab]	1.79±0.4[a]
Sorbitol	33	1,275[ab]	20[a]	1.57±0.9[ab]
Sucrose	43	1,895[a]	12[b]	0.63±0.3[b]

* IVF embryos were cultured in PZM-3 supplemented with 0.05 M sorbitol or sucrose for the first 2 days and then cultured in PZM-3 without sugars for further 4 days. For the control, embryos were cultured in PZM-3 without sugars for whole culture period.

[a,b] Values with different superscripts in the same column differ significantly ($p<0.05$).

tively. More limited ranges of osmolarity are reported for the optimal development of mouse embryos, 250~280 mOsmol for 1-cell (Whitten, 1971), 272~280 mOsmol for 2-cell (Brinster, 1965; Whitten, 1971), and 244~246 mOsmol for rat 1-cell embryos (Miyoshi *et al.*, 1994). In this study, sorbitol and sucrose were compared as a supplement. Osmolarities of PZM-3 and PZM-3 supplemented with 0.05 M sorbitol or sucrose were 256±2.0, 316±3.1 and 315±5.0 mOsmol, respectively. When the NT embryos after fusion were cultured for the first 2 days in the medium supplemented with either 0.05 M sorbitol or sucrose, developmental rate to blastocyst stage was significantly higher ($p<0.05$) in the medium with 0.05 M sucrose than 0.05 M sorbitol. Also, in IVF embryos, developmental rate to blastocyst stage was significantly higher ($p<0.05$) in the medium with 0.05 M sucrose than in the control. In contrast, Im *et al.* (2005) reported that the *in vitro* development of *in vitro* produced porcine NT embryos was greatly enhanced by the addition of sorbitol. The reason for this difference was that culture medium was different for 30 min immediately after fusion. In this experiment, the embryos after fusion were cultured for 30 min in the medium supplemented with either 0.05 M sorbitol or sucrose, but Im *et al.* (2005) transferred the embryos into the PZM-3 immediately after fusion and cultured continuously. Therefore, further studies will be needed to clarify this difference. This result supports the result of the Nguyen *et al.* (2003) who reported that the change of osmolarity of the culture medium after the 4-cell stage increased the rate of expanded blastocyst formation of porcine diploids. The optimal osmolarities of culture medium for the first 2 days were 280~320 mOsmol, and those for the later period were 250~270 mOsmol. Kim *et al.* (2005) also reported that the change of osmolarity from the higher condition (280~300 mOsmol) to the lower condition (256~280 mOsmol) after 2 days of culture can enhance the development of porcine parthenogenetic embryos.

Apoptosis plays an important role in embryo development (Levy *et al.*, 2001; Feugang *et al.*, 2002; Gjorret *et al.*, 2002). Apoptosis occurs during the preimplantation stage in both *in vivo*-and *in vitro*-produced embryos, and it may contribute to embryonic loss. The incidence of apoptosis is higher in bovine blastocysts produced by NT than in embryos produced *in vivo* (Feugang *et al.*, 2002; Gjorret *et al.*, 2002). Research has suggested that a major cause for the level of cell death can be reconciled with the high level of embryos arrest. The generation of healthy zygote is important for understanding the mechanism that causes chromosomal abnormalities during early cleavage stages (Hardy *et al.*, 2001). Apoptosis has been observed in bovine embryos after the 8-cell stage (Neuber *et al.*, 2002). More than 80% of *in vivo* mouse blastocysts on Day 4 or 5 had one or more apoptotic cells (Liu *et al.*, 2002). As in the mouse, the incidence of cell death in the human blastocyst seems to correlate with cell number and embryo quality. The present study showed a similar tendency to the report of Levy *et al.* (2001). They reported that blastocysts with fewer cells had a range of TUNEL-positive cells from 0 to 30%, whereas blastocysts with more cells had less than 10% TUNEL-positive cell. In this study, the early embryos cultured in PZM-3 treated with high osmolarity (300~320 mOsmol) had lower apoptosis rates ($p<0.05$) compared to embryos cultured in PZM-3 of isotonic (250~270 mOsmol). The presence of organic osmolytes such as sorbitol and taurine in the maturation medium improved the microfilament organization at the end of *in vitro* maturation and during early development following IVF of porcine oocytes (Funahashi *et al.*, 1996). In the present study, the quality of NT blastocysts was not better than that of IVF blastocysts. But, the qualities of NT and IVF blastocysts were increased when embryos were cultured in the hypertonic condition at the early embryonic culture stage.

In conclusion, this study represents that the culture of embryos in a high osmolarity culture condition at the early embryonic culture stage for the first 2 days of culture period can improve membrane stabilization resulting in reduced apoptosis.

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