The Effect of Porcine Sperm Cytosolic Factor (SCF) on *In Vitro* Development of Porcine PA and NT Embryos

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ABSTRACT

This study investigated whether the addition of porcine sperm cytosolic factor (SCF) at fusion/activation affects *in vitro* development of porcine parthenogenetic(PA) and nuclear transfer (NT) embryos. To determine the optimum concentration of SCF, control group of oocytes was activated with 0.3M mannitol (1.0 mM $CaCl_2 \cdot 2H_2O$), other three groups of oocytes were parthenogentically activated with the fusion medium (0.1mM $CaCl_2 \cdot 2H_2O$) supplemented with 100, 200 or 300 µg/ml SCF, respectively. Matured oocytes were activated with two electric pulses (DC) of 1.2 kv/cm for 30 µsec. The activated embryos were cultured in PZM-3 under 5% CO_2 in air at 38.5℃ for 6 days. Oocytes activated in the presence of SCF showed a significantly higher blastocyst rate than control ($p<0.05$). Apoptosis rate was significantly lower in 100 µg/ml SCF group than other groups ($p<0.05$). Cdc2 kinase activity in control and SCF treatment group of oocytes was determined using MESACUP cdc2 kinase assay kit at 1, 5, 10, 15, 30, 45 and 60 min after activation. Cdc2 kinase activity was significantly decreased ($p<0.05$) in SCF group than MII oocytes or control within 5 min. For NT embryo production, reconstructed oocytes were fused in the fusion medium supplemented with 0.1 mM $CaCl_2 \cdot 2H_2O$ (T1), 1.0 mM $CaCl_2 \cdot 2H_2O$ (T2) and 0.1 mM $CaCl_2 \cdot 2H_2O$ with 100 µg/ml SCF (T3). Fused embryos were cultured in PZM-3 under 5% CO_2 in air at 38.5℃ for 6 days. Developmental rate to blastocyst stage was significantly higher in T3 than other groups (23.0% vs. 13.5 to 15.2%) ($p<0.05$). Apoptosis rate was significantly lower in T3 than T1 or T2 ($p<0.05$). The relative abundance of Bax-α/Bcl-xl was significantly lower in *in vivo* or SCF group than that of control ($p<0.05$). Moreover, the expression of p53 and caspase3 mRNA was significantly lower in *in vivo* or SCF group than that of control ($p<0.05$). These results indicate that the addition of SCF at fusion/activation might improve *in vitro* development of porcine NT embryos through regulating cdc2 kinase level and expression of apoptosis related genes.

(Key words : SCF, Porcine NT embryo, Developmental ability, Cdc2 kinase activity)

INTRODUCTION

Cloned pigs have a potential application in the fields of xenotransplantation and the production of animal models for human physiology and disease (Prather *et al.*, 2000). Since the first report that reconstructed pig oocytes using fibroblasts could develop to the blastocyst stage *in vitro* (Tao *et al.*, 1999), a number of studies have been conducted to improve the developmental ability of porcine nuclear transfer (NT) embryos (Cheong *et al.*, 2000; Koo *et al.*, 2000; Uhm *et al.*, 2000; Verma *et al.*, 2000; Ikeda and Takahashi, 2001; Lai *et al.*, 2001; Hyun *et al.*, 2003; Im *et al.*, 2005). However, the success rates in cloned pigs are so low that only 1~5% reconstructed embryos produce live offspring. Recently, stud-

ies have been performed to improve the developmental competence of porcine *in vitro* produced embryos (Abeydeera *et al.*, 1998; Kano *et al.*, 1998; Wang *et al.*, 2000; Gandhi *et al.*, 2001) and have demonstrated that many factors are involved in *in vitro* NT embryo development and viability.

Activation is an essential procedure to perform NT successfully due to the absence of the elevation of calcium level induced by sperm penetration into oocytes. It is well documented that at fertilization the first Ca^{2+} transient and some subsequent Ca^{2+} spikes are responsible for egg activation characterized by cortical granule exocytosis leading to blocking of the polyspermy and resumption and completion of the second meiotic division (Kline and Kline, 1992). Later, long-lasting Ca^{2+} oscillation facilitates early embryonic development such as

pronucleus formation (Ducibella *et al.*, 2002; Swann and Ozil, 1994). Oocyte activation can be stimulated parthenogenetically in the absence of the sperm by chemical agents and electric pulses that lead to transient increases in $[Ca^{2+}]_i$ (Whittingham, 1979). Also, recently in most NT studies, porcine NT embryos following fusion have been treated with combined chemicals such as calcium ionophore A23187/cycloheximide or ionomycin/6-MAP (Betthauser *et al.*, 2000). But, Im *et al.* (2007) reported that artificial activation such as electric pulses and combined chemicals caused only one or two rises in $[Ca^{2+}]_i$, unlike the oscillation induced by the sperm, which is not sufficient to activate oocytes.

Machaty *et al.* (2000) demonstrated that the sperm cytosolic extract could induce $[Ca^{2+}]_i$ elevation and oocyte activation. The results indicate that an increase of Ca^{2+} level in the oocytes could be induced by a cytosolic factor existed in the sperm cytosol. Although the mechanism by which the sperm initiates these $[Ca^{2+}]_i$ transients has not been fully elucidated, the several previous reports support the idea that the sperm delivers a soluble factor resulting in the activation shortly after fusion of the gamates (Homa and Swann, 1994; Saunders, 2002; Swann, 1990; Wu *et al.*, 1997).

Therefore, this study was carried out to determine whether the addition of porcine sperm cytosolic factor (SCF) at fusion/activation affects *in vitro* development of porcine NT embryos.

MATERIALS AND METHODS

All chemicals, unless noted otherwise, were from Sigma, St. Louis, MO.

Preparation of SCF

SCF was prepared according to Machaty *et al.* (2000) with some modifications. Crude cytosolic sperm extracts were prepared from boar semen. The sperm-rich fraction of an ejaculate was collected and the semen was centrifuged at 1,300 g for 10 min two times by resuspending spermatozoa with an intracellular-like medium. The intracellular-like medium consisted of 120 mM KCl, 20 mM HEPES, 100 μM ethyleneglycol-bis-(β-aminoethylether)N,N,N',N'-tetraacetic acid (EGTA), 10 mM Na-glycerophosphate, 200 μM phenylmethylsulfonyl fluoride (PMSF) and 1 mM dithiothreitol (DTT) with pH 7.5. The sperm suspension was subjected to a freeze-thaw cycle by submerging it in liquid nitrogen and then thawing it at room temperature. Homogenate was centrifuged at 100,000g for 1 h at 4°C and the supernatant was collected as the cytosolic fraction. It was concentrated using Centricon YM-100 ultrafiltration membranes (Amicon, Inc., Bedford, MA). The total protein

content was determined using BCA protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's protocols. The protein content of the extract was determined spectrophotometrically at 562 nm (Spectrophotometer U-3300, Hitachi, Tokyo, Japan). The final protein concentration was 1,000±200 μg/ml. Protein was aliquoted and stored at -80°C. They were thawed immediately before use.

Collection of Oocytes and *In Vitro* Mmaturation (IVM)

Porcine ovaries were obtained from a local slaughterhouse and transported to the laboratory at 30~35°C. 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 follicular fluid was pooled into 50 ml conical tubes and the sediment was washed in Tyrode's lactate (TL)-Hepes containing 0.1% (w/v) polyvinyl alcohol (PVA). 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 h at 38.5°C 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 luteinizing hormone, 0.5 μg/ml follicle stimulating hormone, 10 ng/ml epidermal growth factor, 75 μg/ml penicillin G and 50 μg/ml streptomycin.

Production of Parthenogenetic (PA) 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. Cumulus-free oocytes with the first polar body were placed between 0.2 mm diameter wire electrodes, 1 mm apart in activation medium. The medium used for activation was 0.3 M mannitol 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 an Electro Cell fusion (NEPA gene, Chiba, Japan). Activated embryos were cultured in PZM-3 under 5% CO_2 in air at 38.5°C (Im *et al.*, 2004).

Preparation of Porcine Fetal Fibroblast Cells

A 35 days porcine fetus was retrieved from the pregnant gilt. After the brain, intestines, and four limbs were removed, tissues were cut into small pieces with fine scissors. The minced cells were incubated for 30 min at 39°C in PBS supplemented with 0.05% trypsin and 0.02 mM EDTA, and the suspension was centri-

fuged at 1,200 rpm for 5 min. Cell pellet were 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 Nuclear Transfer (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 with a glass pipette. Enucleation was confirmed by staining the oocytes with 10 μg/ml Hoechst 33342 for 15~20 min at 39℃. All micromanipulation procedures were performed in TCM-199 supplemented with 3 mg/ml BSA and 5 μg/ml cytochalasin B. After enucleation, the oocytes were held in TCM-199 supplemented with 3 mg/ml BSA until injection of donor cells. Cells were trypsinized and held in TCM-199 supplemented with 3 mg/ml BSA. A single cell with a smooth surface was transferred into the perivitelline space of an enucleated oocyte. Reconstructed oocytes were cultured for 1~2 h in TCM-199 supplemented with 3 mg/ml BSA until fusion. They 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 an Electro Cell fusion (NEPA gene, Chiba, Japan). After fusion treatment, the reconstructed oocytes were cultured in TCM-199 supplemented with 3 mg/ml BSA for 30 min and the fusion was determined. Embryos were washed and transferred into PZM-3 covered with mineral oil in a four-well dish under 5% CO$_2$ in air at 38.5℃.

Apoptosis Assays

The blastocysts on Days 6 from NT were washed twice in PBS/PVP (PBS supplemented with 0.1% polyvinylpyrolidone) and fixed in 4% (v/v) paraformaldehyde solution for 24 h at 4℃. Membranes were permeabilized in 0.5% Triton X-100 for 30 min at room temperature. A TUNEL assay was used to assess the presence of apoptotic cells (in situ cell death detection kit, TMR red; Roche, Mannheim), for 1 h at 38.5℃ in the dark. The broken DNA ends of the embryonic cells were labeled with TDT and fluorescein-dUTP. After the
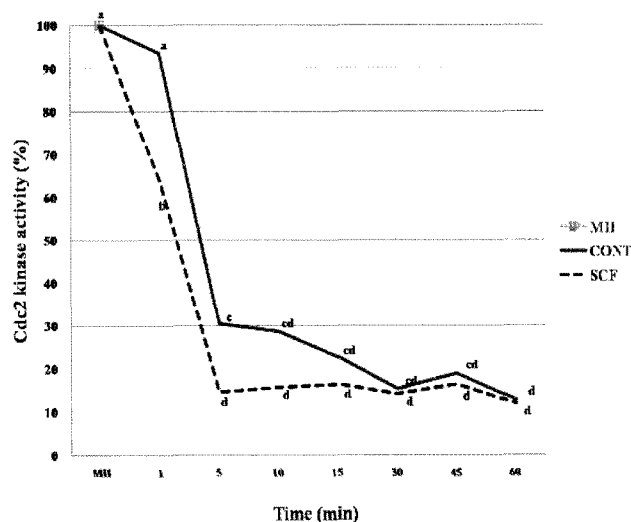

Fig. 1. Change of cdc2 kinase activity of the oocytes after electric activation. MII shows MPF level of 44 h matured oocytes. Control and SCF indicates MPF level of the activated oocytes with two electric pulses (DC) and two DC + SCF, respectively. [a~d] Different superscripts represent statistical differences between treatments ($p<0.05$).

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 anti-fade Kit (cat. P-748, Molecular Probes, Eugene, OR). 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).

Collection of In Vivo Blastocysts

Embryos were collected from synchronized 7 to 11-month-old Landrace gilts. The gilts were artificially inseminated 24 and 36 h after hCG injection. Immediately after stunning and bleeding of the animals, approximately 168 h after hCG (120 h after estimated ovulation), the genital tract was removed and flushed with DPBS for embryo collection. Embryos were transported to the laboratory in PVA-TL-Hepes buffered at 37℃ within 30 min after collection.

RealTime RT-PCR Quantification

The day 6 blastocysts were washed in DEPC-treated water and stored at -70℃. In all experiments, β-actin mRNA was used as an internal standard. First standard cDNA synthesis was achieved by reverse transcription of the RNA by using the Oligo (dT) 15 primer and the 1st strand cDNA synthesis kit (Roche). The mRNAs of Bax-α, Bcl-xl, p53, caspase3 and β-actin were then detected by Realtime RT-PCR with specific primer pairs (Table 1). PCR reactions were performed ac-

Table 1. Primer sequences and cycling condition used in realtime RT-PCR

Gene	Primer sequence (5'-3')	Fragment size (bp)	Realtime RT-PCR condition
p-β Actin	F:CACTGGCATTGTCATGGACT R:GGCAGCTCGTAGCTCTTCTC	285	60°C, 12s 45 cycles
p-Bcl-xl	F:GTTGACTTTCTCTCCTACAAGC R:GGTACCTCAGTTCAAACTCATC	277	62°C, 13s 50 cycles
p-Bax-α	F:ACTGGACAGTAACATGGAGC R:GTCCCAAAGTAGGAGAGGAG	294	63°C, 13s 55 cycles
p-p53	F:AGCAAAAGAAGAAACCACTG R:CCCCTTCTTAGACTTCAGGT	173	63°C, 11s 50 cycles
p-caspase3	F:GAAAATACCAGTTGAGGCAG R:CATGGACACAATACATGGAA	250	63°C, 11s 50 cycles

cording to the instructions of the Realtime PCR machine manufacturer (LightCycler, Roche) and detected with SYBR Green, a double-stranded DNA-specific fluorescent dye included in the SYBR Green PCR premix. Each PCR run was performed in 20 µl reaction buffer containing 10 µl 2× SYBR Green premix, 1 µl of forward, reverse primers (100 pmol/µl), 1 µl embryonic cDNA (0.1 blastocyst/µl equivalent). All samples were measured in triplicate. The following amplification program was employed: preincubation for HotStart polymerase activation at 95°C for 15 min, followed by 40~55 amplification cycles of denaturation at 95°C for 1 min (2°C/sec), annealing at 60~63°C for 1 min (2°C/sec), elongation at 72°C for 1 min (2°C/sec), and acquisition of fluorescence at 72 or 80°C for 1 sec. After the end of the last cycle, the melting curve was generated by starting fluorescence acquisition at 65°C, and taking measurements every 0.2°C until a temperature of 95°C. Product sizes were confirmed by electrophoresis on a standard 2% agarose gel stained with ethidium bromide, and visualized by exposure to ultraviolet light.

Cdc2 Kinase Assay

Maturation promoting factor (MPF) assay was conducted by measuring the activity of its catalytic subunit, cdc2 kinase, with MESACUP cdc2 kinase assay kit (MBL, Nagoya, Japan). The correlation coefficients between cdc2 kinase activity examined by the MESACUP cdc2 kinase assay kit and histon H1 kinase activity measured by the radioactive method were as high as 0.9961. The assay was carried out according to the manufacturer's directions. Briefly, twenty activated oocytes were washed twice with the cdc2 kinase sample buffer containing 50 mM Tris HCl, 0.5 M NaCl, 5 mM EDTA, 2 mM EGTA, 0.01 % Brij35, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.05 mg/ml leupeptin, 50 mM 2-mercaptoethanol, 25 mM β-glycerophosphate and 1 mM Na-orthovanadate. Oocytes were then transferred to a microtube containing 5 µl of the buffer and stored at -80

°C. At the time of assay, oocytes were lysed by freezing and thawing with liquid nitrogen. Five microliters of oocytes extract were mixed with 45 µl kinase assay buffer containing 25 mM Hepes buffer (MBL), 10 mM MgCl$_2$, (MBL), 10 % biotinylated MV peptide (Ser-Lue-Tyr-Ser-Ser-Ser-Pro-Gly-Gly-Ala-Tyr-Cys; MBL) and 0.1 mM ATP and then incubated for 30 min at 30°C. The phosphorylation reaction was terminated by the addition of 200 µl of stop reagent (PBS containing 50 mM EDTA; MBL) and centrifuged for 15 sec at 14,000 g. For the detection of cdc2 kinase by ELISA, each 100 µl of the reaction mixture was transferred to each microwell strip coated with monoclonal antibody recognizing the phosphorylated form of the biotinylated MV peptide. The microwells were incubated at 25 °C for 60 min, and then washed 5 times with washing solution (PBS). One hundred microliters of horseradish peroxidase conjugated streptavidin solution were added to each well and then incubated at 25°C for 30 min. After washing, 100 µl of the POD substrate solution were added, and incubated for additional 5 min. Finally, 100 µl of stop solution (20 % H$_3$PO$_4$) were added to each well, and the optical density of each well read at 492 nm with a microplate reader.

Experimental Designs

Experiment 1 was conducted to investigate the optimum concentration of SCF. Matured oocytes were activated with two electric pulses (DC) of 1.2 kv/cm for 30 µ sec. Control group of oocytes were activated with 0.3 M mannitol (1.0 mM CaCl$_2$·2H$_2$O) and other three groups of oocytes were activated with 0.3 M mannitol (0.1 mM CaCl$_2$·2H$_2$O) supplemented with 100, 200 or 300 µg/ml SCF, respectively. Then, these embryos were cultured in PZM-3 under 5% CO$_2$ in air at 38.5°C. After 6 days of culture, development to blastocyst, total cell numbers and apoptosis in blastocysts were examined. Experiment 2 was designed to determine MPF levels between control and SCF group. Matured oocy-

tes were activated with two electric pulses (DC) of 1.2 kv/cm for 30 μsec and sampled at 0.5, 5, 10, 15, 30, 45 and 60 min, respectively, after activation. Experiment 3 was carried out to investigate the development of porcine NT embryos fused/activated in the presence SCF. Reconstructed oocytes were fused in 0.3 M mannitol supplemented with 0.1 mM $CaCl_2 \cdot 2H_2O$ (T1), 1.0 mM $CaCl_2 \cdot 2H_2O$ (T2) and 0.1 mM $CaCl_2 \cdot 2H_2O$ + 100 μg /ml SCF (T3). Experiment 4 was conducted to examine the expression of apoptosis related genes in porcine NT blastocysts. Day 6 blastocysts derived from NT were analyzed by using realtime RT-PCR analysis.

Statistical Analysis

Data 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). A probability of $p < 0.05$ was considered statistically significant.

RESULTS

Development of PA Embryos Produced in the Presence of SCF

There were no significant differences in the cleavage rate among different treatment groups (68.1 to 76.7%). However, oocytes activated with SCF showed a significantly higher ($p < 0.05$) blastocyst formation rate than control (21.3 to 23.1% vs. 13.2%). The apoptosis rate was significantly lower ($p < 0.05$) in 100 μg/ml SCF than other groups (1% vs. 4 to 6%) (Table 2). ICM/TE ratio was 0.16, 1.24, 0.20, and 0.22, respectively, according to treatments. There were no significant differences in ICM/TE ratio.

Cdc2 Kinase Activity

The activity of cdc2 kinase, the catalytic subunit of MPF, in the cytoplasm was determined. The cdc2 kinase activity was dramatically changed during 5 min after activation. Immediately after electric activation, MPF activity of the oocytes activated in the presence of SCF was significantly decreased ($p < 0.05$) than control and continued to decrease by 5 min. Since 5 min the activity remained low by 1 hr. However there was no difference in the activity between groups from 10 min to 1 hr ($p < 0.05$, Fig. 1).

Table 2. Development of porcine PA embryos produced in the presence of SCF

Treatment* (μg/ml)	No. of oocytes treated	No. of oocytes developed to		% TUNEL
		≥2 cell	Blastocyst	
Control	248	175 (68.1±4.29)	35 (13.2±2.22)[b]	15.4/262 (6±0.01)[a]
SCF 100	255	189 (74.4±1.50)	57 (23.1±2.97)[a]	4.8/557 (1±0.00)[b]
SCF 200	251	186 (74.2±1.57)	56 (21.3±2.10)[a]	22.5/455 (5±0.01)[a]
SCF 300	253	194 (76.7±4.50)	58 (23.0±3.39)[a]	18.7/457 (4±0.01)[a]

* Control group of oocytes were activated with 0.3 M mannitol (1.0 mM $CaCl_2 \cdot 2H_2O$) and other three groups of oocytes were activated with 0.3 M mannitol (0.1 mM $CaCl_2 \cdot 2H_2O$) supplemented with 100, 200 or 300 μg/ml SCF, respectively.
[a,b] Values with different superscripts differ significantly ($p < 0.05$).

Table 3. Development of porcine NT embryos produced in the presence of SCF

Treatment*	No. of oocytes fused/ manipulated	No. of NT embryos cultured	No. (mean±SE) of NT embryos developed to		% TUNEL
			≥2 cell	Blastocyst	
T1	336/417	336	256 (79.1±0.03)	44 (13.5±0.01)[b]	140/1436 (10.5±0.01)[a]
T2	371/404	371	296 (84.4±0.02)	53 (15.2±0.01)[b]	110/1502 (8.2±0.01)[a]
T3	391/439	391	314 (83.1±0.02)	86 (23.0±0.01)[a]	122/2097 (6.4±0.01)[b]

* 0.1 and 1.0 mM $CaCl_2 \cdot 2H_2O$ were supplemented in T1 and T2, respectively. For T3, 0.1 mM $CaCl_2 \cdot 2H_2O$ and 100 μg/ml SCF were supplemented.
[a,b] Values with different superscripts differ significantly ($p < 0.05$).

Development of NT Embryos Produced in the Presence of SCF

There was no difference among treatments in cleavage rate, but developmental rate to the blastocyst stage was significantly higher ($p<0.05$) (23.0% vs. 13.5% to 15.2%) in SCF group (T3) than other groups. The apoptosis rate was significantly lower ($p<0.05$) in SCF group (T3) than other groups (6.4% vs. 10.5% to 8.2%) (Table 3). ICM/TE ratio was 0.26, 0.22, and 0.25, respectively, according to treatments. There were no significant differences in ICM/TE ratio.
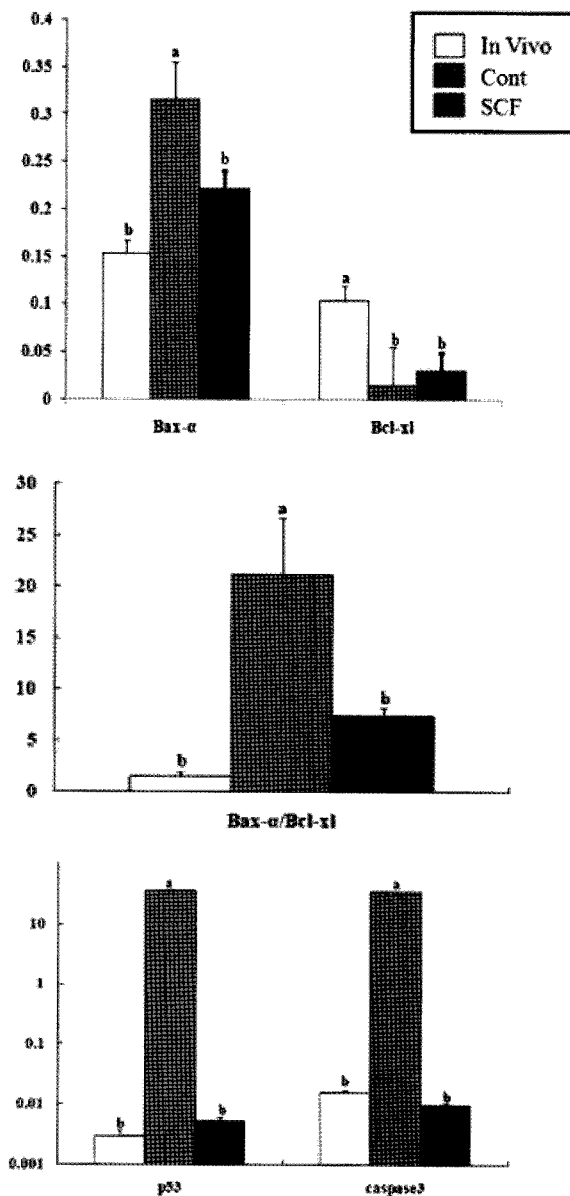

Fig. 2. Relative Bax-α, Bcl-xl, Bax-α/Bcl-xl, p53 and caspase 3 mRNA expression in NT derived blastocyst. Day 6 blastocysts derived from NT and *in vivo* were analyzed by using realtime RT-PCR analysis. [a,b]Different superscripts represent statistical differences between treatments ($p<0.05$).

Expression of Apoptosis-Related Genes in NT Embryos

The relative abundance of Bax-α mRNA expression was significantly lower in SCF or *in vivo* group than control ($p<0.05$). For Bcl-xl, control or SCF groups showed lower mRNA expression than *in vivo* ($p<0.05$). The relative abundance of Bax-α/Bcl-xl was clearly lower in *in vivo* or SCF group than control ($p<0.05$). Also, the relative abundance of p53 and caspase3 mRNA expression was significantly higher in control than that of *in vivo* or SCF group ($p<0.05$, Fig. 2).

DISSCUSSION

The present study shows that the addition of SCF at fusion/activation medium can improve subsequent development to the blastocyst stage with better quality. Development of porcine PA (21.3% to 23.1% vs. 13.2%) and NT embryos (23.0% vs. 13.5% to 15.2%) fused/activated in the presence of SCF was increased significantly ($p<0.05$). Both porcine PA and NT embryos were improved in developmental rate with enhanced embryo quality. Generally, cell fusion and activation are induced simultaneously by electric pulses in 0.3 M mannitol containing 0.1mM $CaCl_2 \cdot 2H_2O$. However, Cheong *et al.* (2002) demonstrated that increasing Ca^{2+} concentration to 1.0 mM $CaCl_2 \cdot 2H_2O$ in 0.3 M mannitol could improve the fusion and blastocyst formation rates of porcine NT embryos without an additional activation treatment. In the present results, the NT embryos fused/activated with 0.1 mM $CaCl_2 \cdot 2H_2O$ and 100 μg/ml SCF showed significantly higher development to the blastocyst stage in comparison with the embryos fused/activated with 1.0 mM $CaCl_2 \cdot 2H_2O$ (23.0% vs. 15.2%).

In natural condition, the ovulated oocyte is arrested at metaphase II stage and cannot resume the meiosis without fertilization. The meiotic arrest is maintained by the high level of MPF which is stabilized by c-mos or cytostatic factor (CSF). In oocytes of all mammalian species, fertilization by a sperm induces a series of species-specific increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) that is crucial for oocyte activation (Fissore *et al.*, 1992; Kline and Kline, 1992). This fertilization-associated oscillation is responsible for down regulation of maturation promoting factor (MPF), which allows resumption of the second meiotic cell division, cortical granule exocytosis, block to polyspermy, pronuclear formation and initiation of embryonic cleavage (Kline and Kline, 1992; Carroll, 2001). MPF is responsible to start of oocyte maturation as a complex formed by cyclin B and cdc2 kinase (Gautier *et al.*, 1990; Yamashita *et al.*, 1992). MAP kinase is also activated during oocyte maturation (Shibuya *et al.*, 1992; Verlhac *et al.*, 1994). A crucial role of MAP kinase in oocyte maturation seems

to be to control the levels of cyclin B. MAP kinase is essential for the induction of metaphase arrest and CSF, both of which require large Ca^{2+} transients for release. Paradoxically, MAP kinase inactivation following fertilization is necessary to allow MPF and completion of the first mitotic cell cycle. (Abrieu et al., 2005). Generally, MPF is activated at GVBD and increase until it reaches a plateau at the end of the first meiotic M-phase (Choi et al., 1991; Verhac et al., 1994). A transient decrease in MPF activity takes place during the conversion between meiosis I and II. MPF is reactivated rapidly to enter meiosis II and is stayed at a high level during the metaphase II arrest. (Brunet and Maro, 2005). A high level of MPF activity that causes nuclear membrane breakdown (NEBD), spindle assemble and premature chromosome condensation (PCC). After fertilization, activated oocyte induces decay of MPF activity and chromosome segregation, chromosome decondensation and centrosome diplication. After DNA replication, the cell divides to produce a 2-cell embryo. We monitored MPF activity following different oocyte activation protocols. Our data demonstrated that the addition of SCF into fusion/activation medium induced faster MPF inactivation. It indicates that SCF might trigger the similar activation, which is induced by a sperm, and improve the developmental ability of porcine PA and NT embryos.

It is well known that environmental stress; such as in vitro culture system and activation condition induces unscheduled apoptosis in cultured embryos (Jurisicova et al., 1998; Byrne et al., 1999). Apoptosis may contribute to the progressive loss of embryos during the pregnancy. TUNEL assay for detecting apoptosis revealed the cell with apoptosis in PA and NT blastocyst. The PA (1% vs. 4% to 6%) and NT (6.4% vs. 8.2%) blastocysts produced in SCF treatment showed a significantly lower apoptosis rate than that of other groups. In order to verify the apoptosis of blastocyst, we analyzed the apoptosis related genes (Bax- α, Bcl-xl, p53 and caspase 3). Entry to and progression through the apoptotic pathway seem to be controlled by the balanced expression of several conserved genes that have either pro- or anti- apoptotic effects. The Bcl-2 gene family is known to include anti- and pro-apoptotic subgroups, and Bcl-xl gene functions to protect against apoptosis (Boise et al., 1993). In contrast, another group of highly conserved genes are positive regulators of apoptosis, which includes the Bax- α protein in the pig. The tumor suppressor p53 was an important mediator of responses to cellular stress (Lane and Hall, 1997) and caspase, a family of cysteine proteases, is crucial in mediating apoptosis. The activation of caspase-3 that belongs to the caspase effector is the last step of the apoptotic cascade, leading to the cleavage of cellular substrates important for cell survival (Cryns and Yuan, 1999). In the present study, the relative abundance of Bax- α/Bcl-xl

was clearly lower in in vivo or SCF groups than control. Also, the relative abundance of p53 and caspase3 mRNA expression was significantly higher in control than that of in vivo or SCF groups, suggesting that SCF might increase survival factors by blocking apoptotic pathways.

In conclusion, this study demonstrates that the addition of SCF at fusion/activation might improve the in vitro development of porcine NT embryos through regulating MPF activity and the expression of apoptosis related genes.

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