Effect of Porcine Follicular Fluid on Donor Cell Characteristics and Quality of Porcine Cloned Blastocysts

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ABSTRACT

This study aimed at investigating whether a porcine follicular fluid (pFF) supplementation positively affects the characteristics of donor cells and the developmental competence of porcine cloned embryos. Ear fibroblast cells (donor cell) from an Massachusetts General Hospital miniature pig were cultured in different culture methods: (1) Dulbecco's modified Eagle's medium (DMEM)+10% FBS (Control); (2) DMEM+0.5% FBS (SS); and (3) DMEM+10% FBS+10% pFF (pFF) for 72 h. In each conditioned medium, the concentrations of 4 amino acids (Thr, Glu, Pro, and Val) in the pFF group were significantly different from those in the control group (p<0.05 or p<0.01). The proliferation of the cells cultured in the SS group was significantly lower than that of the other treatment groups (p<0.01). The population of apoptotic and necrotic cells in the SS group was significantly higher than that of either the control or the pFF group (p<0.01). The number of embryos that cleaved (p<0.05) and developed into blastocysts (p<0.01) in the SS group was significantly lower than that of either the control or the pFF group. Compared to other groups, the blastocysts produced from the donor cells in the pFF group had higher total cells and lower apoptotic cells (p<0.05). It can be concluded that pFF supplementation in the donor cell culture medium positively affects cell death, cell cycle and quality of the cloned blastocyst.

(Key words : Porcine follicular fluid, Amino acids, Cell cycle, Cloned embryo development)

INTRODUCTION

Somatic cell nuclear transfer (SCNT) technique has been developed to produce transgenic cloned (TG-NT) animals for xenotransplantation research (Lai *et al.*, 2002; Murakami *et al.*, 2002; Takahagi *et al.*, 2005; Ahn *et al.*, 2011). However, the efficiency of TG-NT animal production is still very low regardless of species.

To increase the cloning efficiency, many techniques such as *in vitro* culture system (Yoon *et al.*, 2000; Egerszegi *et al.*, 2010; Min and Park, 2012); egg manipulation for gene injection (Klose *et al.*, 2005; Park *et al.*, 2006) or SCNT (Im *et al.*, 2007; Park *et al.*, 2011); and molecular biology for gene cloning, TG donor cell establishment, or analysis of embryo quality (Shim *et al.*, 2009), have been used. And also one of the most important factors for the efficiency is donor cell quality.

Oocyte, follicular cells, and embryo are being grown in a hormonal regulation environment in the female reproductive organs such as oviduct and uterus. Unlike these reproductive cells, however, somatic donor cells are not (or are rarely) exposed to reproductive hormones before its reconstruction and transfer into the uterus.

Follicular fluid (FF) contains growth factors, electrolytes, amino acids, various unknown factors, and several reproductive hormones (Abeydeera *et al.*, 2000; Dode and Graves, 2002; Hong *et al.*, 2004; Iwata *et al.*, 2004). The supplementation of porcine FF (pFF) in *in vitro* maturation (IVM) medium was reported to improve oocyte maturation, monospermic fertilization, and embryonic development (Algriany *et al.*, 2004; Hong and Lee, 2007; Metoki *et al.*, 2008). However, little is known about an effect of pFF supplementation on the characteristics of somatic donor cells.

Therefore, this study was performed to investigate the effect of pFF supplementation in donor cell culture medium on the proliferation and apoptosis induction of somatic cells, as well as on the developmental competence of cloned embryos.

MATERIALS AND METHODS

Preparation of Cumulus-Oocyte Complexes (COCs) and FF

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Porcine ovaries were obtained from a local slaughterhouse (Pyoung-Nong Corp., Pyoungtaek, Gyeonggi, Korea) and were transported to the laboratory at 36℃. Cumulus-oocyte complexes were collected and washed in Tyrode's lactate-Hepes containing 0.1% (w/v) polyvinyl alcohol (PVA). The oocytes with several layers of cumulus cells were selected and washed 3 times in TCM-199 (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 0.1% PVA, 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 0.57 mM cysteine, 0.5 μg/ml LH, 0.5 μg/ml FSH, 10 ng/ml EGF, 10% pFF, 75 μg/ml penicillin G, and 50 μg/ml streptomycin. For *in vitro* maturation, 50~60 COCs were transferred into 0.5 ml of maturation medium in a 4-well dish (Nunc, Roskidle, Denmark). The oocytes were matured for 20 h (with hormones)+20 h (without hormones) at 38.7℃ with 5% CO_2 in air. The FF collected was centrifuged for 20 min (10,000 g) at 4℃. The supernatant was collected, filtered (0.2 μm), and stored at −20℃ until further use.

Isolation and Culture of Miniature Pig Ear Fibroblasts

Ear tissue was obtained from a 2-year-old female Massachusetts General Hospital miniature pig (MGH minipig) at the National Institute of Animal Science. The tissue was washed 3 times in PBS (Invitrogen) and minced with a surgical blade. The minced cells were incubated for 30 min at 39℃ in PBS supplemented with 0.05% trypsin and 0.02 mM EDTA, and the suspension was centrifuged at 1,200 rpm for 5 min. The cell pellets were resuspended and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) supplemented with 15% FBS and 75 μg/ml antibiotics at 38.7℃ with 5% CO_2 in air. After the removal of the unattached cells, the attached cells were cultured to confluence and then either trypsinized for further passage or stored in liquid nitrogen. For these experiments, the cells were cultured by 3 different methods for 72 h: control (DMEM+10% FBS), SS (DMEM+0.5% FBS), and pFF (DMEM+10% FBS+10% pFF).

Determination of Amino Acid Concentrations

For an analysis of the amino acid concentrations in the conditioned medium, the samples were acid-hydrolyzed using 6 N HCl for 24 h at 110℃ and were measured by an automatic amino acid analyzer (Hitachi L-8800, Japan) at 254 nm after reaction with PITC (phenylisothiocyanate) derivative reagent (White *et al.*, 1988). A C18 reversed phase column (Spherisorb S3 ODS2, 4.6 ×150 mm, Waters, Bio-Lab, USA) was used for separation. A mobile phase was run with 50 mM ammonium acetate in acetonitrile and methanol gradients for over 40 min.

Cell Proliferation Assay

A cell proliferation assay was evaluated by using tetrazolium salt (WST1, TAKARA Bio, Japan). The cells were plated onto 96-well microtiter plates (Nunc) at a seeding density of 5×10^3 cells in a volume of 100 μl per well and then cultured for 72 h at 38.7℃ under 5% CO_2 in air. The cell proliferation assay was performed according to the manufacturer's instructions. On the day of the assay, 10 μl of WST1 was added and then the cells were incubated for an additional 2 h under the same conditions. The wells containing the cells with 10% FBS served as the control. The optical density was measured on a microtiter plate reader (Bio-Rad, Hercules, CA, USA) at 440 nm. The data are expressed as percentages of the absorbance readings from the control wells.

Determination of Apoptotic and Necrotic Cell Distribution

To identify apoptotic cells, the cells were stained with propidium iodide (PI) and fluorescein isothiocyanate (FITC)-conjugated annexin V using the Annexin V-FITC Apoptosis Detection Kit (BioVision, USA), following the manufacturer's instructions. The cells were detached with 0.05% trypsin, washed with ice-cold PBS, and resuspended in a binding buffer (5×10^5 cells/ml). Then, the cells were centrifuged at 1,000 rpm for 5 min at 4℃. After the supernatant had been discarded, the cells were resuspended in 500 μl of binding buffer with the addition of 5 μl of annexin-V-FITC and 5 μl of PI. Subsequently, after incubation in the dark (20 min on ice), the cell preparations were immediately analyzed by flow cytometry using a Beckman Coulter Cytomics FC-500 cell sorter with FITC fluorescence (Ex=488 nm; Em=530 nm). According to the FACS data, the cells were divided into 3 groups: Annexin V(+)/PI(−) as apoptotic cells, Annexin V(−)/PI(+) as necrotic cells, and Annexin V(−)/PI(−) as normal cells.

SCNT Procedure

After *in vitro* maturation, cumulus cells were treated with PBS supplemented with 0.1% PVA and 0.1% hyaluronidase for 4 min. The oocytes with the first polar body were enucleated and stained with 10 μg/ml Hoechst 33342 for confirmation of enucleation. The donor cell from each of the 3 different culture methods was transferred into the perivitelline space of one of the enucleated oocytes. After 1 h of equilibration time, the reconstructed oocytes were placed into the 0.2 mm diameter wire electrodes (1 mm apart) of a fusion chamber covered with 0.3 M mannitol solution containing 0.1 mM $MgSO_4$, 1.0 mM $CaCl_2$, and 0.5 mM Hepes. For fusion, 2 DC pulses (1.5 kV/cm) were applied for 30 μsec using a BTX 2001 Electro Cell Manipulator (BTX, Holliston, MA, USA). The fused embryos were washed and then transferred into PZM-3 at 38.7℃ under 5% CO_2 and 5% O_2 in air. Cleavage and blastocyst development was examined at day 2 and day 6, respec-

tively.

TUNEL Assay for Apoptotic Cell Death

Blastocysts at day 6 were washed twice in PBS/PVP (0.1% polyvinylpyrrolidone) and fixed in 4% paraformaldehyde solution for 24 h at 4℃. After permeabilization using 0.5% Triton X-100, the apoptotic cells were analyzed by an *in situ* cell death detection kit (TMR red, Roche, Indianapolis, IN, USA) for 1 h at 39℃ in the dark. The embryos were stained with 10 μg /ml Hoechest 33342 for 30 min and mounted on slides with a Prolong antifade Kit (Molecular Probes, Eugene, OR, USA). The slides were stored at −20℃. The number of total and apoptotic cells was determined from optical images of whole-mount embryos under an epifluorescent microscope (Nikon, Japan).

Statistical Analysis

Data were subjected to a Generalized Linear Model Procedure (PROC-GLM) of the Statistical Analysis System (SAS Institute). Differences among treatment means were determined by using the Duncan's multiple range test. All data are expressed as Least Square (LS) mean (SD). A probability of $p<0.05$ was considered significantly significant.

RESULTS

Measurement of Amino Acid Concentration

The concentrations of amino acids in the conditioned medium obtained from the 3 different culture methods are shown in Table 1. For the pFF group, the concentration of Thr ($p<0.01$), Glu ($p<0.05$), and Pro ($p<0.05$) was significantly higher and that of Val ($p<0.05$) was significantly lower than that of those from the other groups. In the SS group, the level of Val ($p<0.05$) and Cys ($p<0.05$) was significantly higher and the concentration of Glu ($p<0.01$) was significantly lower than that of those from the other groups.

Cell Proliferation Assay

As we expected, the proliferation of the cells cultured in the SS group was significantly lower than that of the other treatment groups ($p<0.01$) (Fig. 1). The donor cells cultured in the pFF-supplemented medium showed higher proliferation ability compared with the donor cells of the control group, although the difference was not significant.

Determination of Apoptotic and Necrotic Cells

The population of apoptotic and necrotic cells in the SS group was significantly higher than that of either the control or the pFF group ($p<0.01$) (Fig. 2). In the

Table 1. Determination of amino acid concentrations in the conditioned medium

Amino acids	Control	SS	pFF
THR	57.4±2.37	50.7±3.68	72.9±6.26**
HIS	9.77±0.22	9.87±0.51	9.53±1.18
I-LE	30.8±2.65	30.9±0.99	26.4±2.79
LEU	32.0±2.81	33.3±1.02	29.3±3.18
LYS	42.1±5.24	44.1±8.93	40.8±23.2
MET	8.97±0.38	9.07±0.31	8.25±0.74
PHE	17.6±0.58	17.9±0.48	16.4±1.5
VAL	33.8±1.16	39.7±1.79*	27.2±2.91*
ARG	18.9±0.69	18.5±0.44	16.9±1.73
ASP	0.36±0.06	0.43±0.06	0.57±0.29
CYS	10.2±0.37	18.1±1.57**	9.04±0.75
GLU	17.2±3.26	12.9±1.55*	25.1±5.27*
GLY	11.2±0.35	11.2±0.4	11.3±1.13
PRO	1.13±0.14	1.19±0.28	1.90±0.48*
SER	15.3±0.30	15.1±0.7	13.4±1.03
TYR	22.5±0.66	21.9±0.58	20.1±2.05

The concentration (*u*M) of amino acids was measured by an automatic amino acid analyzer (Hitachi L-8800) at 254 nm. Data were expressed as mean±standard deviation (SD) of 3 independent experiments.
* : $p<0.05$; ** : $p<0.01$: within a row.

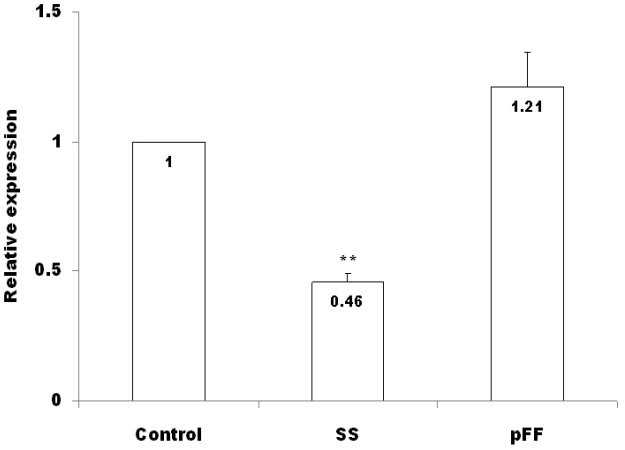

Fig. 1. Results obtained by WST-1 assay to describe cell proliferation after 3 days culture. The cells were cultured with DMEM containing 10% FBS (control); 0.5% FBS (SS); and 10% FBS+10% pFF (pFF) for 3 days, respectively. The data were presented as relative expression (divided by control) and mean±standard deviation (SD) of 3 independent experiments. ** $p<0.01$.

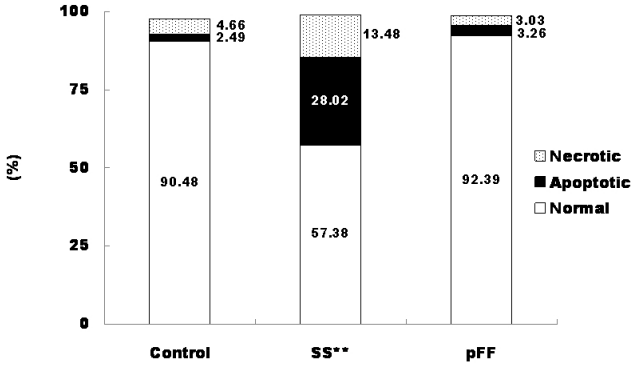

Fig. 2. Apoptotic and necrotic population of ear fibroblast cells of NIH MHC Inbred miniature pig. The cells were cultured with DMEM containing 10% FBS (control); 0.5% FBS (SS); and 10% FBS+10% pFF (pFF) for 3 days, respectively. Percentage of apoptotic and necrotic cells was detected using Annexin-V FITC/PI staining by flow cytometry. ** $p<0.01$.

Table 2. *In vitro* developmental competence of cloned embryos

	No. (%) of embryos		
	Reconstructed	≥ 2cell	Blastocyst
Control	245	194 (79.2±1.8)	62 (25.3±2.2)
SS	165	122 (73.9±5.9)*	25 (15.1±2.9)**
pFF	198	160 (84.3±4.7)	57 (28.8±2.2)

The cells were cultured with DMEM containing 10% FBS (control); 0.5% FBS (SS); and 10% FBS+10% pFF (pFF) for 3 days, respectively.
Data were expressed as mean±SD of 3 independent experiments.
* : $p<0.05$; ** : $p<0.01$: within a column.

pFF supplementation group, it was shown a tendency of a higher level of normal cells and a lower level of necrotic cells compared to the control group.

Developmental Competence of Cloned Embryos

The number of embryos that cleaved ($p<0.05$) and developed into blastocysts ($p<0.01$) in the SS group was significantly lower than that of either the control or the pFF group (Table 2), respectively. No significant cleavage and developmental differences were found between the control and the pFF group.

Apoptotic Cell Death in Cloned Blastocysts

The number of total and apoptotic cells in the cloned blastocysts is presented in Table 3. The blastocysts that originated from the donor cells cultured in the pFF supplemented medium had a significantly higher number of total cells and a significantly lower number of apoptotic cells than the control group ($p<0.05$). On the other hand, the embryos that developed into blastocysts in the SS group had a lower number of total cells and a higher number of apoptotic cells than the control group ($p<0.05$). The apoptotic cells were significantly higher in the SS group ($p<0.01$) and significantly lower in the pFF group ($p<0.05$) than that in the control group.

Table 3. Effects of pFF on the quality of cloned blastocysts

	No. of			Ratio# (%)
	Blastocysts	Total cells	Apoptotic cells	
Control	16	31.3±3.4	1.6±0.3	5.1±1.6
SS	17	23.7±3.1*	3.7±1.2*	15.3±2.9**
pFF	17	37.3±4.0*	0.9±0.3*	2.5±0.6*

The cells were cultured with DMEM containing 10% FBS (control); 0.5% FBS (SS); and 10% FBS+10% pFF (pFF) for 3 days, respectively.
Data were expressed as mean±SD of 3 independent experiments.
* : $p<0.05$; ** : $p<0.01$: compared to the control group.
# : Apoptotic cells / total cells × 100

DISCUSSION

For *in vitro* maturation and development of porcine eggs, various culture media such as TCM-199, NCSU-23, or PZM-3 with or without growth factors and hormones were used. For donor cells, however, one of the most common culture medium is DMEM containing 10% FBS without any additional supplementations.

To keep cells alive for longer periods, the basal medium must be supplemented with several factors. Among these factors, serum is most commonly used for the maintenance and proliferation of cells. Fetal bovine serum (FBS) contains a large number of components, such as growth factors, proteins, vitamins, trace elements, etc., that are essential for the growth and maintenance of cells (van der Valk *et al.*, 2010). Serum, however, is not a good supplier of AAs to endothelial cells in culture and cell growth can be significantly affected by altering the AA composition of the culture medium (Gorman *et al.*, 1996).

Nakazawa *et al.* (1997) reported that the concentration of AAs in FF was around 60% of that in human serum. Based on this report, a supplementation of 10% pFF brings about almost a 6% increase in the total AA concentration of the culture medium. In the present study, the concentrations of 4 AAs (Thr, Glu, Val, and Pro) in the pFF group and the concentrations of 3 AAs (Val, Cys, and Glu) in the SS group changed significantly after 3 days of culturing. Interestingly, the concentration of AAs that were differently determined in the SS group showed a distinct opposite expression pattern when compared to that of those in the pFF group.

According to the donor cell proliferation and apoptotic population results, the proliferation rate of cells in the SS group was significantly lower and the population of apoptotic and necrotic cells was significantly higher than that of those in the control group. The supplementation of pFF in the donor cell culture medium, however, improved the rate of donor cell proliferation and increased the number of normal cells. The proliferation rate in the pFF group was 20% higher than that of the control group and 50% higher than that of the SS group. Interestingly, the concentration of glutamate (Glu) in the pFF group was also about 30～50% higher than that of the other treatment groups. Glutamate has a number of important functions, as it plays an important role in preserving high-energy phosphates in muscle. Moreover, intracellular Glu is known to be an important precursor for antioxidant glutathione and glutamine synthesis (Engelen *et al.*, 2000). Thus, factors which include AAs may positively affect both the proliferation and maintenance of donor cells.

To increase the efficiency of producing cloned animals, several cell cycle synchronization methods such as serum starvation (Wilmut *et al.*, 1997) and confluency (Nagashima *et al.*, 2003) have been used. Many cloned animals were produced using G0 stage of donor cells by the serum starvation. However, Stice *et al.* (2000) proposed that serum starvation that is mostly adapted for the synchronization of nuclear donor at G0/G1 phase might increase the occurrences of apoptosis and necrosis of the cells, leading to inappropriate DNA replication or DNA damage.

The exposure of the donor cells into the pFF affects not the development of the cloned blastocyst, but the quality of the blastocysts. The pFF provides a special microenvironment containing regulatory molecules and proteins that play a pivotal role in the embryo quality and reproductive process (Lédée *et al.* 2008; Jarkovska *et al.*, 2010). In this study, the blastocysts that originated from the donor cells cultured with the pFF-supplemented medium had a significantly higher number of total cells and a significantly lower number of apoptotic cells than that of those in the control group. Environmental stress caused by *in vitro* culture systems and activation conditions induce unscheduled apoptosis in cultured embryos (Jurisicova *et al.*, 1998; Byrne *et al.*, 1999). It can be postulated that factors such as growth factors, hormones, AAs, etc., in FF may contribute to stabilizing the microenvironment of the culture medium and to improving the quality of the cloned blastocysts.

Taken together, it can be said that a supplementation of pFF in the donor cell culture medium positively affects the characteristics of the donor cells and the quality of porcine cloned blastocysts.

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