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Supplementation with spermine during in vitro maturation of porcine oocytes improves early embryonic development after parthenogenetic activation and somatic cell nuclear transfer[1]

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ABSTRACT: Spermine plays an important role in protection from reactive oxygen species (ROS) in bacteria, yeast, and mammalian cells, but there are few studies on the effects of spermine on porcine oocyte maturation and subsequent embryo development. The aim of this study was to determine the effects of spermine on in vitro maturation (IVM) of porcine oocytes and their developmental competence after parthenogenetic activation (PA) and somatic cell nuclear transfer (SCNT). We evaluated nuclear maturation, intracellular glutathione (GSH), and ROS levels in oocytes, and their subsequent embryonic development, as well as gene expression in mature oocytes, cumulus cells, and PA blastocysts. After treatment with various concentrations of spermine in IVM culture medium, there was no significant difference in nuclear maturation rate. However, spermine treatment groups (10- 500 μM) showed significantly increased intracellular GSH levels and decreased ROS levels compared to the control ($P < 0.05$). Furthermore, 10 μM spermine supported significantly higher blastocyst formation rates after PA than the control group ($P < 0.05$). According to the optimal condition from the PA results, we investigated the effects of 10 μM spermine on SCNT, and it also significantly improved blastocyst formation rates compared with the control group ($P < 0.05$). In evaluating the effects of 10 μM spermine on gene expression, there was significantly lower expression of a proapoptotic gene (*Bax*) and higher expression of an antiapoptotic gene (*Bcl2*) in cumulus cells ($P < 0.05$). *FGFR2* was increased in spermine-treated oocytes. Levels of transcription for *POU5F1* and *Bcl2* were significantly increased in PA blastocysts. In conclusion, 10 μM spermine supplementation during IVM improved the development of porcine PA and SCNT embryos by increasing intracellular GSH, scavenging ROS levels, and regulating gene expression.

Key words: antioxidant, embryo development, parthenogenetic activation, porcine oocyte, somatic cell nuclear transfer, spermine

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INTRODUCTION

To date, porcine somatic cell nuclear transfer (**SCNT**) has become a useful tool for basic biomedical research and xenotransplantation studies (Yeom et al., 2012; Park et al., 2014). While the first successful SCNT was achieved more than a decade ago, mammalian embryos still have low in vitro developmental competence compared to those following in vivo development (Yang et al., 2007). Oocytes and embryos are vulnerable to adverse factors such as free radicals during in in vitro maturation (**IVM**) and in vitro culture (**IVC**). It is well known that oxidative stress, mainly

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caused by reactive oxygen species (**ROS**), impairs in vitro production (**IVP**) of porcine embryos (Choi et al., 2013). Consequently, antioxidants are required as a defense mechanism for cells to support their function in oxidative environments. Various antioxidants such as resveratrol (Lee et al., 2015), vitamin C (Hu et al., 2012), vitamin E (Tareq et al., 2012), and melatonin (Rodriguez-Osorio et al., 2007) have been added to IVM culture media to improve the capability of porcine oocytes to develop into preimplantation embryos. Spermine, known as polyamine, is a polybasic molecule ubiquitous in all living organisms and cells (Polticelli et al., 2012). It plays important roles in many cellular biochemical and physiological events including the regulation of transcription, modulation of kinase activities, functioning of protein synthesis, activity of ion channels (Pegg, 2014), and providing protection from oxidative damage in mammalian cells (Chattopadhyay et al., 2006). However, none of the past studies elucidated the effects of spermine supplementation on porcine oocyte maturation and subsequent development of porcine parthenogenetic activation (**PA**) and SCNT embryos. Therefore, the current study was designed to investigate the effect of spermine on nuclear maturation, intracellular levels of glutathione (**GSH**) and ROS in mature oocytes, embryonic development after PA and SCNT, and gene expression in oocytes, cumulus cells, and PA-derived blastocysts.

MATERIALS AND METHODS

The protocol for animal use was approved by the Institutional Animal Care and Use Committee of Seoul National University (SNU-141120-8) in accordance with the Guide for the Care and Use of Laboratory Animals of Seoul National University.

Oocyte Collection and In Vitro Maturation

Porcine ovaries were obtained from prepubertal gilts at a local slaughterhouse and transported to the laboratory within 3 h in physiological saline at 32°C–35°C. The contents of follicles (3–6 mm in diameter) were recovered by aspiration with an 18-gauge needle. Cumulus-oocyte complexes (**COC**) were selected and washed 3 times with tissue culture medium-199 (Invitrogen, Carlsbad, CA), 5 mM sodium hydroxide, 2 mM of sodium bicarbonate, 10 mM N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES), 0.3% polyvinyl alcohol (PVA), and 1% Pen-Strep (Invitrogen). Then, 50 COC were placed into IVM medium containing TCM-199 supplemented with 0.57 mM cysteine, 0.91 mM sodium pyruvate, 5 μL/mL insulin transferrin selenium solution (ITS-A) 100X (Invitrogen), 10 ng/mL epidermal growth factor (**EGF**), 10% porcine follicular fluid (vol/vol), 10 IU/mL equine chorionic gonadotropin (**eCG**), and 10 IU/mL human chorionic gonadotropin (**hCG**). The selected COC were incubated at 38.5°C under 5% CO_2 in 95% humidified air for IVM. Following 21–22 h of maturation with hormones, the COC were washed twice in fresh hormone-free IVM medium and then cultured in hormone-free IVM medium for an additional 21–22 h.

Evaluation of Porcine Oocyte Maturation

After 44 h of IVM, cultured oocytes were denuded by gently pipetting with 0.1% hyaluronidase in Tyrode's albumin lactate pyruvate (**TALP**) medium with HEPES buffer, and then denuded oocytes were stained with 5 μg/mL of bisbenzimide (Hoechst 33342) in TALP-HEPES. The stained oocytes were evaluated using a fluorescence microscope (Nikon Corp., Tokyo, Japan). The experiment was repeated 3 times.

Measurement of Intracellular ROS and GSH Levels

Following IVM culture, oocytes at the metaphase II (**MII**) stage were sampled in medium supplemented with different concentrations of spermine or without it for determination of their intracellular ROS and GSH levels. Briefly, H_2DCFDA (2',7'-dichlorodihdrofluorescein diacetate; Invitrogen) and CellTracker Blue CMF_2HC (4-chloromethy-6.8-difluoro-7-hydroxycoumarin; Invitrogen) were used to detect the intracellular ROS level as green fluorescence and the GSH level as blue fluorescence. Each treatment group was incubated (in the dark) for 30 min in Dulbecco's phosphate-buffered saline (**DPBS**; Invitrogen)-PVA containing 10 μM H_2DCFDA and 10 μM CellTracker Blue. After washing 3 times with DPBS, oocytes were placed into 4 μL droplets of TALP-HEPES, and fluorescence was observed under an epifluorescence microscope (TE2000-S; Nikon) with UV filters (460 nm for ROS and 370 nm for GSH). Fluorescence intensities of the oocytes were analyzed using Image J software (Version 1.49q; National Institutes of Health, Bethesda, MD) and normalized to control embryos.

Parthenogenetic Activation of Oocytes

Cumulus-oocyte complexes were denuded by gently pipetting with 0.1% hyaluronidase and washed in TALP medium after 44 h of IVM culture. Denuded oocytes with homogeneous cytoplasm were selected and then gradually equilibrated in activation solution consisting of 0.28 M mannitol, 0.5 mM HEPES, 0.1 mM $CaCl_2$, and 0.1 mM $MgSO_4$. The oocytes were

transferred to a chamber with 2 electrodes spaced 3.2 mm apart that was filled with activation solution and activated by electric stimulation with a single direct current (**DC**) pulse of 1.5 kV/cm for 60 μs utilizing a BXT Electro-Cell Manipulator 2001 (BXT Inc., San Diego, CA). Activated oocytes were washed 3 times in porcine zygote medium-5 (PZM-5; Funakoshi Corporation, Tokyo, Japan), which is serum-free, placed into 30 μL PZM-5 droplets covered with mineral oil, and incubated under a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 38.5°C for 7 d.

Donor Cell Preparation

Ear fibroblasts were isolated from the tissue of an adult pig. Tissues were cut into small pieces and cultured at 38°C in an atmosphere of 5% CO_2 in air in Dulbecco's modified Eagle's medium (**DMEM**; Gibco, culture medium) containing 10% fetal bovine serum (**FBS**; Gibco, culture medium; vol/vol), 1 mM sodium pyruvate, and 100 IU/mL each of penicillin and streptomycin. Cells between passages 4 and 8 were used as donors for SCNT. A single cell suspension was prepared by standard trypsinization immediately before SCNT.

Somatic Cell Nuclear Transfer

After 44 h of IVM culture, matured oocytes with a first polar body (**PB**) were denuded and cultured in PZM-5 medium supplemented with 0.05 mol/L sucrose and 0.4 μg/mL demecolcine for 30 min. Treated oocytes were transferred to TALP-HEPES medium supplemented with 5 μg/mL cytochalasin B (**CB**), 0.05 mol/L sucrose, and 0.4 μg/mL demecolcine, and then the first protruding PB and chromatin plate were removed by aspiration with a glass pipette that had an inner diameter of 15 μm. A single donor cell was inserted into the perivitelline space of an enucleated oocyte, and the couplets were equilibrated with fusion solution (0.28 M mannitol solution containing 0.5 mM HEPES and 0.1 mM $MgSO_4$) for 4 min, and then fused in a 20 μL droplet of fusion solution with a single DC pulse of 200 V/mm for 30 μs using an electrical pulsing machine (LF101; Nepa Gene, Chiba, Japan). After 30 min, fused couplets were equilibrated with activation solution (0.28 M mannitol solution containing 0.5 mM HEPES, 0.1 mM $CaCl_2$, and 0.1 mM $MgSO_4$) for 4 min, transferred to a chamber containing 2 electrodes overlaid with activation solution, and activated with a single DC pulse of 1.5 kV/cm for 60 μs using a BTX ElectroCell Manipulator 2001 (BTX Inc.). SCNT embryos were washed 3 times with fresh PZM-5, and transferred into 30 μL IVC droplets covered with mineral oil, and then cultured at 38.5°C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2. To count total cell numbers of blastocysts, they were collected on d 7, washed in DPBS-PVA, and stained with 25 μg/mL of Hoechst 33342 for 10 min. After a final wash in DPBS-PVA, the blastocysts were mounted on glass slides in a drop of 100% glycerol, compressed gently with a cover slip, and observed under a fluorescence microscope.

Quantitative Real-Time PCR

For the gene expression study, isolated cumulus cells and matured oocytes derived from 250 COC, and 30 PA-derived blastocysts from the 10 μM spermine treatment and the control group, were separately sampled using a stereomicroscope. All samples were washed twice with DPBS and stored at −80°C until RNA was extracted. The total RNA was extracted using TRIzol reagent (Invitrogen), according to the manufacturer's protocol, and the total RNA concentration was quantified using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Complementary DNA (**cDNA**) was produced using Clontech (Takara Inc., Otsu, Japan) in a 10 μL reaction volume. Briefly, 1 μg of total RNA was placed in 0.5 mL PCR tubes, and then 2 μL of 5X RT Master Mix was added. Nuclease-free water (**NFW**; Ambion, Austin, TX) was added to the tubes to adjust the total volume to 10 μL. The tubes were incubated at 37°C for 15 min, then at 50°C for 5 min, and finally heated to 98°C for 5 min. A PCR plate (Micro-Amp Optical 96-Well Reaction Plate, Singapore) was made by adding 1 μL cDNA, 0.4 μL (10 pmol/μL) forward primer, 0.4 μL (10 pmol/μL) reverse primer, 10 μL SYBR Premix Ex Taq (TaKaRa, Otsu, Japan), and 8.2 μL of NFW. The reactions were performed for 40 cycles, and the cycling parameters were as follows: denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. All oligonucleotide primer sequences are presented in Table 1. The expression of each target gene was quantified relative to that of the internal control gene (*GAPDH*) using the equation $R = 2^{-[\Delta Ct\ sample - \Delta Ct\ control]}$. For ease of comparison, the average expression level of each gene from control group was set as 1.

Statistical Analysis

Each experiment was repeated at least 3 times. The data are expressed as the mean values ± standard error of the mean (**SEM**). The data were analyzed using univariate analysis of variance (**ANOVA**) followed by Duncan's multiple range test using SPSS 17.0 (SPSS, Inc., Chicago, IL) statistical software. Differences in gene expression and SCNT blastocyst rates were compared by Student's *t* test. $P < 0.05$ was considered statistically significant.

Table 1. Primer sequences used for real-time PCR[1]

Gene	Primer sequences (5'–3')	Product size, bp	GenBank accession number
GAPDH	F: GTCGGTTGTGGATCTGACCT R: TTGACGAAGTGGTCGTTGAG	207	NM_001206359
POU5F1	F: TTTGGGAAGGTGTTCAGCCAAACG R: TCGGTTCTCGATACTTGTCCGCTT	198	NM_001113060
FGFR2	F: ATTCTGGTGCCGGATGAAGAC R: GGTGTTGGAGTTCATGGAGG	121	NM_001099924
Bax	F: TGCCTCAGGATGCATCTACC R: AAGTAGAAAAGCGCGACCAC	199	XM_003127290
Bcl2	F: AGGGCATTCAGTGACCTGAC R: CGATCCGACTCACCAATACC	193	NM_214285

[1]F, Forward primer; R, Reverse primer.

RESULTS

We evaluated the porcine oocyte maturation rates, GSH and ROS levels, and embryo developmental competence of oocytes at various concentrations of spermine treatment after PA, and development of SCNT embryos and gene expression of oocytes, cumulus cells, and PA-derived blastocyts after IVM treatment with 10 uM spermine.

Effect of Spermine on Nuclear Maturation

In this part, we evaluated the effect of different concentrations (0, 10, 100, 500 and 1000 μM) of spermine on oocyte nuclear maturation by measuring the rate of the first PB extrusion (Fig. 1). A total of 469 oocytes were assessed in 3 replicates, and nuclear maturation rate ranged from 86.3% to 92.8%. However, there were no significant differences among the spermine treatment groups (Table 2).

Effect of Spermine on Intracellular Levels of GSH and ROS

The results (Fig. 2) showed that intracellular levels of GSH in spermine-treated oocytes were significantly higher (levels for the 10, 100, and 500 μM spermine groups were 1.28 ± 0.02, 1.23 ± 0.02, and 1.17 ± 0.03 pixels/oocyte, respectively) than that of the control group (1.00 ± 0.04). Consistent with this finding, levels of ROS were significantly lower in the spermine-treated oocytes (levels for the 10, 100, and 500 μM spermine groups were 0.70 ± 0.05, 0.80 ± 0.04, and 0.82 ± 0.05 pixels/oocyte, respectively) than that of the control group (1.00 ± 0.07 pixels/oocyte).

Effect of Spermine in IVM on Embryonic Development after PA and SCNT

We examined the effect of spermine treatment during IVM on subsequent development of PA embryos. As shown in Table 3, the rate of blastocyst formation was significantly higher in the 10 μM spermine-treated group than in the control group (27.0% vs. 17.6%). However, spermine treatment (10 μM) did not affect cleavage rate (82.2% vs. 78.0%) and total cell numbers per blastocyst (51.6 ± 2.3 vs. 56.2 ± 8.2) in PA embryos.

According to the optimal concentration shown in Table 3, oocytes were treated with 10 μM spermine during IVM and subsequent development of SCNT embryos was compared with the control group. Table 4 shows a significant increase in blastocyst formation and total cell numbers in the treatment group compared with the control group (19.9% and 58.0 ± 14.3 vs. 11.0% and 41.2 ± 8.0, respectively).

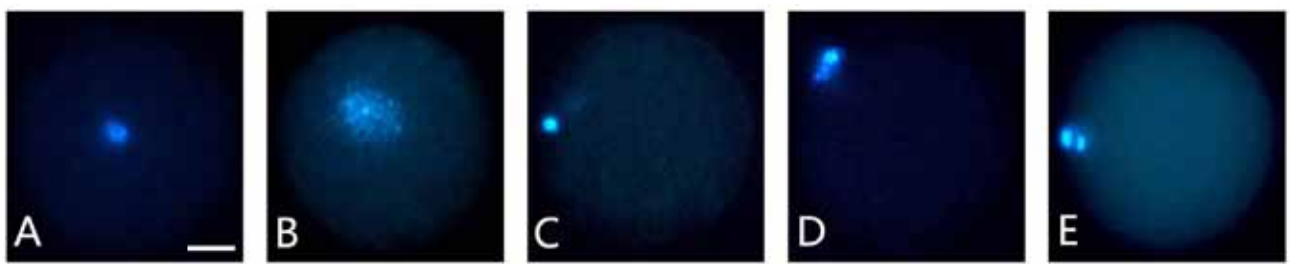

Figure 1. Chromatin configuration of porcine oocytes stained with Hoechst 33342 after 44 h of in vitro maturation. (A) Germinal vesicle. (B) Germinal vesicle breakdown. (C) Metaphase I. (D) Anaphase to Telophase I. (E) Metaphase II. Scale bar indicates 40 μm.

Table 2. Effect of spermine treatment during in vitro maturation (IVM) on nuclear maturation

Spermine concentration, μM	No. of oocytes cultured for maturation[1]	No. (mean ± SEM, %) of oocytes at the stage of			
		GV-GVBD[2]	MI[3]	Ana-Telo[4]	MII[5]
0	88	0 (0.0 ± 0.0)	3 (3.5 ± 2.0)	4 (4.5 ± 2.9)	81 (92.1 ± 2.2)
10	98	0 (0.0 ± 0.0)	6 (5.7 ± 3.1)	2 (2.2 ± 1.1)	90 (92.1 ± 2.3)
100	84	0 (0.0 ± 0.0)	5 (5.7 ± 2.0)	1 (1.3 ± 1.3)	78 (93.0 ± 1.8)
500	97	0 (0.0 ± 0.0)	6 (6.3 ± 0.6)	6 (5.9 ± 3.6)	85 (87.8 ± 3.4)
1,000	102	2 (2.2 ± 2.2)	7 (6.7 ± 2.3)	5 (4.6 ± 4.6)	88 (86.5 ± 3.0)

[1]Experiment was replicated 3 times.

[2]GV-GVBD, Germinal vesicle–Germinal vesicle breakdown.

[3]MI, Metaphase I.

[4]Ana-Telo, Anaphase-Telophase I.

[5]MII, Metaphase II.

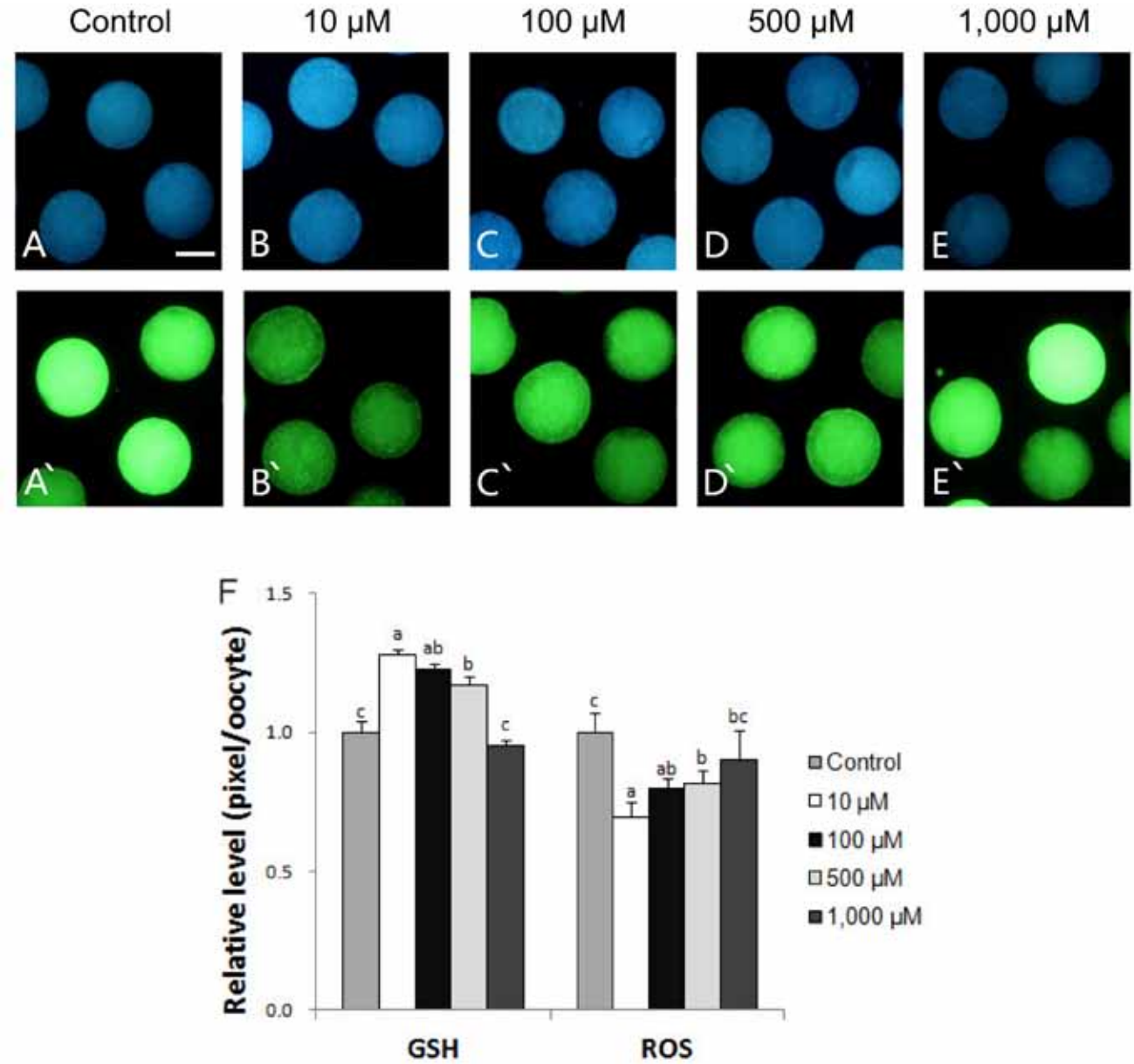

Figure 2. Epifluorescent photomicrographic images of in vitro matured porcine oocytes. Oocytes were stained with CellTracker Blue (A–E) and 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) (A`–E`) to detect intracellular levels of glutathione (GSH) and reactive oxygen species (ROS), respectively. MII oocytes derived from control IVM and various concentration spermine supplemented IVM system. Effect of spermine supplementation during in vitro maturation (IVM) on intracellular GSH and ROS levels in mature oocytes (F). Bars with different superscripted letters (a, b, and c) in each column indicate statistically significant differences (GSH or ROS; $P < 0.05$). Scale bar indicates 100 μm. The experiment was replicated 3 times.

Table 3. Effect of spermine supplementation during in vitro maturation (IVM) on embryonic development after parthenogenetic activation (PA)

Spermine concentration, μM	No. of embryos cultured[1]	No. of embryos developed to (mean ± SEM, %)		Total cell No. (mean ± SEM) in blastocysts
		≥ 2-cells	Blastocyst	
0	165	127 (77.4 ± 3.3)	29 (17.9 ± 3.0)[b]	56.2 ± 8.2
10	174	143 (82.1 ± 2.0)	47 (27.2 ± 1.6)[a]	51.6 ± 2.3
100	173	139 (79.6 ± 3.4)	43 (24.4 ± 2.6)[a,b]	48.8 ± 4.2
500	176	141 (79.7 ± 3.2)	34 (19.1 ± 2.8)[a,b]	51.3 ± 6.1
1,000	164	111 (67.6 ± 8.4)	28 (17.7 ± 3.1)[b]	52.7 ± 5.6

[1]Experiment was replicated 6 times.

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

Gene Expression in Mature Oocytes, Cumulus Cells, and PA-Derived Blastocysts after IVM with Spermine

We evaluated the effect of spermine on *POU5F1*, *FGFR2*, *Bax*, and *Bcl2* gene expression in mature oocytes, cumulus cells, and PA-derived blastocysts. As shown in Fig. 3, 10 μM spermine increased *FGFR2* mRNA transcript levels significantly in oocytes, but not in cumulus cells. In cumulus cells, mRNA transcript levels of *Bax* and *Bcl2* were significantly different in the 10 μM spermine treatment group compared with the control group. Transcript levels for *POU5F1* and *Bcl2* were significantly higher in PA blastocysts derived from 10 μM spermine-treated oocytes than in the control group.

DISCUSSION

Spermine belongs to polyamine and exerts various biological activities, including DNA synthesis, modulation of intracellular signal pathways, cell proliferation, and differentiation (Porat and Clark, 1990). As spermine play antioxidant function by direct scavenging of ROS, this is a very active area with biochemical and physiological studies using mammals, plant, protozoan parasites, microorganisms, and anti-inflammatory agents (Lovaas, 1995; Pegg, 2009). This is substantiated by the rapid induction of ornithine decarboxylase (DOC) by oxidative stress. Spermine is contained in the epidermis at high levels with a 30 times stronger antioxidant effect than vitamin E, and thereby delays skin aging by 20% (Lovaas, 1995). Therefore, it is one of the natural antioxidant cosmetic ingredients in anti-aging creams (ORYZA, 2012). As a natural antioxidant, spermine added to blood preservative solutions provides better red blood cell storage and "survival" (Kucherenko and Bernhardt, 2015). In addition, a decrease in spermine directly influences embryonic development or embryonic cell blocks at early stages in mice (Nishimura et al., 2002).

In this study, we demonstrated that spermine treatment during IVM had a beneficial effect on cytoplasmic maturation and subsequent development of PA and SCNT embryos. Supplementation with 10 μM spermine during IVM increased GSH levels and decreased ROS levels in mature oocytes. Furthermore, 10 μM spermine treatments significantly improved subsequent in vitro development of PA and SCNT embryos. Expression of some genes was also positively changed in mature oocytes, cumulus cells, and PA blastocyst.

Intracellular GSH is one of the major antioxidants, and it plays a pivotal role in maintaining redox homeostasis, scavenging peroxides, and detoxifying xenobiotics (Hayes et al., 2005). ROS plays an important role in modulating an entire spectrum of events in reproductive physiology, such as oocyte maturation, fertilization, embryo development, and pregnancy (Agarwal et al., 2005). However, it is generally accepted that high levels of ROS have multiple adverse effects on mitochondria and nuclei, and can cause oxidative stress, change the membrane lipid composition, and decrease the cellular concentration of ascorbic acid and the ratio of intracellular GSH/GSH disulfide (Gardiner et al., 1998; Tarín et al., 2002). Intracellular GSH is used as a molecular marker that predicts cytoplasmic maturation in porcine oocytes. Therefore, we speculated that the influence on GSH might result from

Table 4. Effect of spermine treatment during in vitro maturation (IVM) on embryonic development after somatic cell nuclear transfer (SCNT)

Spermine concentration, μM	No. of embryos cultured[1]	No. of embryos developed to (mean ± SEM, %)		Total cell no. (mean ± SEM) in blastocysts
		≥ 2-cells	Blastocyst	
0	209	163 (79.6 ± 12.4)	23 (11.0 ± 1.0)[b]	41.2 ± 8.0[b]
10	196	151 (77.7 ± 16.0)	39 (20.2 ± 3.4)[a]	58.0 ± 14.3[a]

[1]Experiment was replicated 3 times.

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

changes in intracellular ROS levels in porcine oocytes. In our experiment, after culturing COC in maturation media supplemented with spermine, although there were no significant differences in oocyte maturation rates, the levels of GSH with the addition of 10 μM spermine were higher than that in other groups, and the expression of ROS was decreased.

To investigate the effect of spermine on development and apoptosis of oocytes and cumulus cells, we analyzed gene expression of *POU5F1 FGFR2*, *Bax*, and *Bcl2*. Transcription factor *POU5F1* is an essential gene for early development that is overexpressed in good-quality oocytes and oocyte-derived blastocysts (Kwak et al., 2012). *FGFR2* is the primary receptor partner for the oocyte competent factor, such as FGF10 and FGF7, and its signaling is involved in regulating oocyte maturation, cumulus expansion, and subsequent embryonic development (Zhang et al., 2010). Our study showed that *FGFR2* gene expression of the 10 μM spermine treatment group was significantly higher than the control group of mature oocytes. *Bax* is a proapoptosis gene and *Bcl2* is an antiapoptosis gene (Lowthert et al., 2012). *Bax* expression was reduced significantly, and *Bcl2* expression was significantly higher in cumulus cells derived from the 10 μM spermine-treated group. These results showed that spermine treatment reduced apoptosis in cumulus cells.

The beneficial effects of spermine as an antioxidant also contribute to embryo developmental competence, and this was reflected in blastocyst formation rates. The addition of 10 μM spermine to IVM medium increased blastocyst rates significantly after PA, but not in high concentration (500–1,000 μM). It is consistent with the previous study showing that a relatively high concentration of exogenous spermine may induce cell death (Brunton et al., 1990). Therefore, with all the results, the optimal concentration for porcine oocyte maturation and embryo development was established as 10 μM. According to a previous study, spermine addition to the IVC medium did not enhance embryo developmental competence or the total number of nuclei after PA. The 10 μM concentration of polyamines in IVC reduced the percentage of blastocysts developing after PA (Cui and Kim, 2005). However, based on our results, 10 μM spermine treatments during IVM not only significantly improved blastocyst formation rates after both PA and SCNT, but also significantly increased the total cell numbers in SCNT blastocysts. Moreover, *POU5F1* expression was significantly increased in PA blastocysts after 10 μM spermine supplementation in IVM. The *Bcl2* antiapoptotic transcript expression in blastocysts from the 10 μM spermine treatment group was also upregulated. Therefore, upregulated *POU5F1* and *Bcl2* expression was reflected in increased blastocyst formation of PA embryos. Our results are consistent with previous studies showing that spermine could enhance the development of in vitro fertilization (**IVF**) embryos (Porat and Clark, 1990) and may act to prevent the generation of free radicals soon after IVF and protect the embryos from oxidative damage at the early stage (Lovaas and Carlin, 1991; Natsuyama et al., 1992).

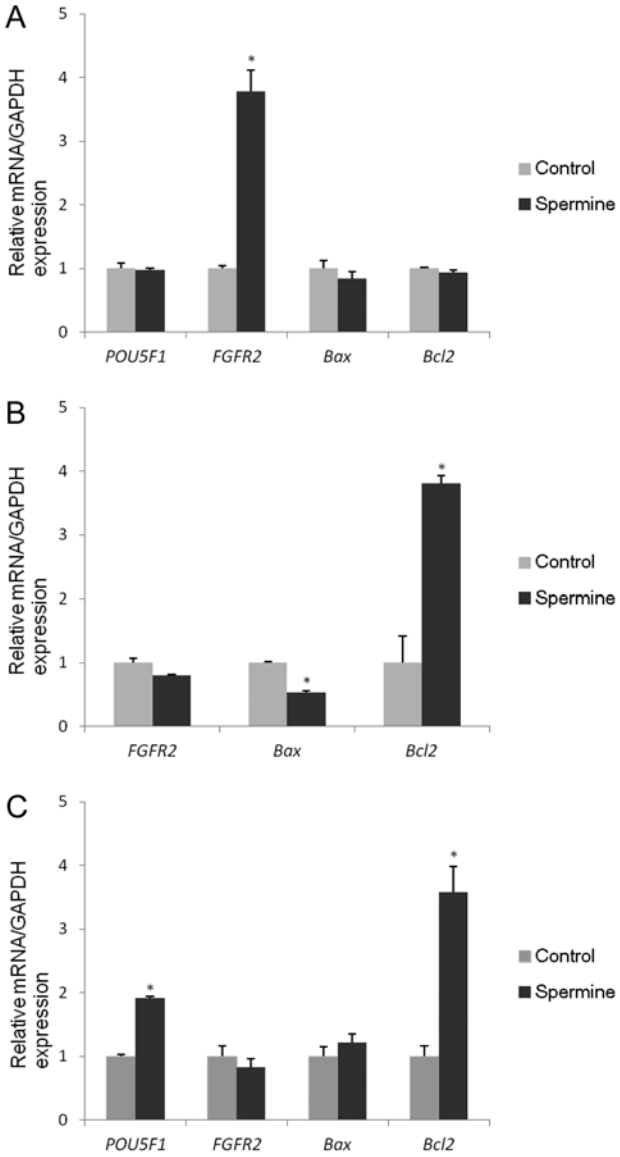

Figure 3. The mRNA expression levels (mean ± SEM) of *POU5F1*, *FGFR2*, *Bax*, and *Bcl2* in mature oocytes (A), cumulus cells (B), and PA-derived blastocysts (C) after 10 μM spermine supplementation during in vitro maturation. Within the same mRNA, bars with an asterisk are significantly different ($P < 0.05$). The experiment was replicated at least 3 times.

The present study focused on the application of optimal conditions with spermine treatment during oocyte IVM on the in vitro development of porcine embryos. Based on our findings, we propose that treatment of porcine oocytes with 10 μM spermine has a beneficial effect on preimplantation development leading to enhanced PA and SCNT blastocyst formation rates by increasing the intracellular GSH levels, decreasing the

ROS levels, and regulating gene expression related to development (*POU5F1* and *FGFR2*) and apoptosis (*Bax* and *Bcl2*). In the future, to use spermine routinely for the enhancement of in vitro conditions for mammalian oocytes and embryos, additional experiments such as terminal deoxynucleotidyl transferase dUTP nick end labeling (**TUNEL**) assay for detecting DNA fragmentation and Western blotting assay of apoptosis-regulating proteins are required.

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