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Effect of Vascular Endothelial Growth Factor on *in vitro* Porcine Oocyte Maturation and Subsequent Developmental Competence after Parthenogenesis

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Abstract: We investigated the effects of Vascular Endothelial Growth Factor (VEGF) on *in vitro* maturation and subsequent embryos developmental competence after Parthenogenesis (PA) and Somatic Cell Nuclear Transfer (SCNT). For this porcine, Cumulus Oocyte Complexes (COCs) were matured in the medium supplemented with different concentrations of Vascular Endothelial Growth Factor (VEGF) and then the maturation and intracellular Glutathione (GSH) concentration of oocytes were examined. In addition, the developmental competence of oocytes matured with different concentrations of VEGF after Parthenogenetic Activation (PA) or Somatic Cell Nuclear Transfer (SCNT) was observed. Although, the maturation rates among these groups were not significantly different (81.13±2.61; 83.93±1.97; 82.14±4.03; 75.24±2.68, respectively). Total intracellular Glutathione (GSH) concentrations of oocytes matured with 5-50 ng mL^{-1} VEGF were increased significantly (12.68±0.076 and 12.33±0.53 pMol $oocyte^{-1}$, respectively) compared to the control and 500 ng mL^{-1} (10.19±0.66 and 10.54±0.54 pMol $oocyte^{-1}$) groups. The blastocyst formation rates after PA of oocytes matured with 5-50 ng mL^{-1} VEGF were increased significantly (58.99±4.70 and 50.00±1.09%, respectively) compared with the control and 500 ng mL^{-1} VEGF (30.15±4.52 and 34.79±4.01%, respectively). Total cells number were significantly higher in 5-50 ng mL^{-1} VEGF treatment groups (83.21±4.89 and 78.16±6.15, respectively) compared to control and 500 ng mL^{-1} VEGF treated groups (56.91±4.78 and 55.93±3.89, respectively). Similarly, the blastocyst formation rate and total cell number (14.54±1.42, 67.83±6.56, respectively) after SCNT of oocytes matured with 5 ng mL^{-1} VEGF was significantly higher than that of oocytes matured without VEGF (7.95±1.44 and 48.09±5.36, respectively). The rate of COCs with fully expanded cumulus was significantly higher in 5 ng mL^{-1} VEGF treated group (85.37±0.73%) compared to control (58.89±0.88%). In conclusion, adding 5 ng mL^{-1} VEGF during IVM improved the developmental potential of PA and SCNT in porcine embryos by increasing the intracellular GSH level during oocyte maturation.

Key words: VEGF, glutathione, parthenogenesis, SCNT, porcine oocytes, maturation

INTRODUCTION

Cytoplasmic maturation is an important factor for successful fertilization and embryonic development. Optimal cytoplasmic maturation helps pronuclear formation which is important for developing an embryo. In general, cytoplasmic maturation involves the accumulation of mRNA, proteins, substrates and nutrients that are required to achieve oocyte developmental competence that fosters embryonic developmental competence (Watson, 2007). Among these, Glutathione (GSH) has important biological functions during cellular proliferation, amino acid transportation, protein and DNA synthesis and reduction of disulfide bonds (Meister and Anderson, 1983). Cytoplasmic GSH is an index of cytoplasmic maturation (De Matos and Furnus, 2000; Eppig, 1996) and plays an important role in protecting not only somatic cells (Meister, 1983) but also mammalian gametes (Luberda, 2005). Ntracellular GSH levels and the ability of the cytoplasm to decondense the sperm nucleus or to induce male pronuclear formation have been used as indices to evaluate cytoplasmic maturation. Cytoplasmic GSH is regulated by cumulus cells during *In vitro* Maturation (IVM) (Maedomari *et al*., 2007). However,

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increased intracellular GSH levels frequently promote male pronuclear formation after fertilization (Sun and Nagai, 2003). Vascular Endothelial Growth Factor (VEGF) is a homodimer composed of two subunits, each with a molecular mass of 23 kDa (Gospodarowicz *et al*., 1989). Currently, VEGF includes 7 members: VEGF-A, -B, -C, -D, -E, -F and phosphatidylinositol-glycan biosynthesis class F protein. All members have a common VEGF homology domain. VEGF is important for cell proliferation in normal and tumor cells and it is able to promote cell differentiation in some cells (Diaz-Cueto and Gerton, 2001). In the female reproductive system, VEGF is essential for follicular and corpus luteum development and as a valuable biochemical marker of oocyte maturation (Findlay, 1986; Kawano *et al*., 2003). VEGF acts via two tyrosine kinase family receptors, namely flt-1 (VEGFR-1) and flk-1/KDR (VEGFR-2) (Ferrara and Davis-Smyth, 1997; Shibuya, 1995).

However, evidence indicates that adding VEGF to oocyte maturation medium could increase the blastocyst rate in the bovine IVM system (Luo *et al*., 2002a) and that the action is mediated by cumulus cells (Luo *et al*., 2002b), suggesting that VEGF has some initial beneficial effects during embryo development but the exact mechanism is not yet clear and inadequately investigated with porcine COCs.

During Somatic Cell Nuclear Transfer (SCNT), nuclear reprogramming is essential for developing an embryo *in vitro* which requires good cytoplasmic maturation and is desired for effective and successful SCNT embryonic production (Solter, 2000; Nandedkar *et al*., 2009).

Good quality cytoplasm is essential to support 1-3 embryonic cycles of newly introduced nuclear DNA in the absence of embryonic transcripts or during nuclear reprogramming and thus may affect gene expression during nuclear reprogramming (Dominko *et al*., 1999). In this study, we investigated whether oocytes matured under VEGF have some beneficial effects during parthenogensis, as VEGF is capable of inducing protein synthesis during oocyte maturation which could aid in subsequent development.

MATERIALS AND METHODS

Ovary collection, recovery and *in vitro* oocyte maturation: Ovaries of prepubertal gilts were collected from a commercial abattoir and transported to the laboratory within 2 h in 0.9% (w/v) NaCl solution supplemented with penicillin-G (100 U mL^{-1}) and streptomycin sulfate (100 mg mL^{-1}) at 30-35°C. The follicular fluid with oocytes was aspirated from 3-7 mm antral follicles with a 10 mL disposable syringe and 20-gauge needle and collected in a 15 mL conical tube. Cumulus-Oocyte Complexes (COCs) were recovered under a stereoscope microscope; those with at least three layers of compact cumulus cells and with homogenous cytoplasm were selected for IVM. The selected COCs were transferred and cultured in 500 μL of tissue culture medium 199 (Life Technologies, Rockville, MD, USA) supplemented with 26 mM sodium bicarbonate, 0.91 mM sodium pyruvate, 0.57 mM cysteine, 10 ng mL^{-1} epidermal growth factor, 0.5 IU mL^{-1} porcine luteinizing hormone, 0.5 IU mL^{-1} porcine follicle stimulating hormone, 10% (v/v) pFF, 75 μg mL^{-1} penicillin-G and 50 μg mL^{-1} streptomycin.

The COCs were then statically cultured at 39°C in a humidified atmosphere containing 5% CO_2 with 10I U mL^{-1} eCG (Intervet International BV). After 20-22 h of maturation with hormones, the oocytes were washed twice in a fresh maturation medium before being cultured in hormone-free medium for additional 18 h for SCNT and 22 h for parthenogenesis. The pFF was aspirated from 3-7 mm follicles of prepubertal gilt ovaries and were prepared according to Hyun *et al*. (2003) and stored at -20°C until use.

Micromanipulation for scnt, fusion and activation: Fibroblasts were isolated from fetuses at day 40 of gestation. The head and other soft tissues were removed using iris scissors and watchmaker's forceps and discarded. After washing twice with DPBS (Invitrogen, Carlsbad, CA, USA), the carcass was minced with a surgical blade on a 100 mm culture dish. The minced fetal tissues were dissociated in DMEM (Invitrogen) supplemented with 0.1% (w/v) trypsin and 1 mM EDTA (Invitrogen) for 1-2 h.

Trypsinzed cells were washed once by centrifugation at 300× g for 10 min and subsequently seeded into 100 mm plastic culture dishes. Seeded cells were then cultured for 6-8 days in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS; Invitrogen), 1 mM sodium pyruvate, 1% (v/v) non-essential amino acids (Invitrogen) and 10 mg mL^{-1} penicillin-streptomycin solution at 39°C in a humidified atmosphere of 5% CO_2 and 95% air. After removing the unattached clumps of cells or explants, the attached cells were further cultured until confluent. Subculturing was done at intervals of 5-7 days by trypsinization for 2 min using 0.1% trypsin and 0.02% EDTA.

The cells were then stored in freezing medium in liquid nitrogen after two passages. The freezing medium consisted of 70% (v/v), DMEM, 10% (v/v) DMSO and 20% (v/v) FBS. Prior to SCNT, the cells were thawed and subsequently cultured in 10% FBS with DMEM. The

donor cells were synchronized at the G0/G1 stage of the cell cycle by contact inhibition for 3-4 days. The individual cells were retrieved from the monolayer by trypsinization for ~1 min and subsequently used for SCNT. After 44 h of IVM, COCs were transferred to HEPES-buffered NCSU-23 medium containing 0.5 mg mL^{-1} hyaluronidase (Sigma; St. Louis, MO, USA) for 1 min; the cumulus cells were subsequently removed by gentle pipetting.

Cumulus-cell-free oocytes were incubated for 2 min in a manipulation medium (calcium free TLH-BSA) containing 5 μg mL^{-1} Hoechst 33343 (Sigma). Following incubation, the oocytes were transferred into a drop of manipulation medium and were overlaid with warm mineral oil. The zona pellucida was partially dissected with a fine glass needle to make a slit near the first Polar Body (PB). The first PB and adjacent cytoplasm (~10%), presumably containing the metaphase-II (M-II) chromosomes were extruded by squeezing the oocytes with the same needle. Enucleation was confirmed under an epifluorescence microscope (TE 300, Nikon, Tokyo, Japan). Using a fine injecting pipette, a 12-15-μm trypsinized fetal fibroblast with a smooth cell surface was transferred into the periviteline space through the same slit of an enucleated oocyte.

The couplets were equilibrated with 0.28 M mannitol solution containing 0.5 mM HEPES, 0.1 mM $CaCl_2$ and 0.05 $MgSO_4$ for 2-3 min and transferred to a fusion chamber containing two electrodes overlaid with mannitol solution. Membrane fusion and activation were induced by applying an Alternating Current (AC) field of 2 V cycling at 1 MHz for 2 sec followed by two pulses of 160 V mm^{-1} Direct Current (DC) for 50 μ sec using a cell fusion generator (LF101; NepaGene, Chiba, Japan).

Activated oocytes were washed 3-4 times with NCSU-23 medium supplemented with 4 mg mL^{-1} fatty-acid free BSA and placed in humidified incubator at 39°C under 5% CO_2. After 1 h, the fusion was checked and fused, properly shaped oocytes were washed 3-4 times and further cultured with NCSU-23 medium covered with prewarmed mineral oil and then incubated under 5% O_2, 5% CO_2 and 90% N_2 at 39°C for 168 h. For Parthenogenetic Activation (PA), the oocytes with PB at 44 h of IVM were activated using a pulse sequence identical to that used to activate SCNT oocytes. Post activation culture was same as SCNT oocytes. At day 4 in both *in vitro* Cultures (IVC), medium was supplemented with 10% FBS (final concentration) (Invitrogen, CA).

M-II oocytes evaluation and staining: The effect of supplementation of different concentration VEGF on maturation was assessed by the maturation rate at 42 h of IVM. All cumulus cells were completely removed by gentle pipetting with 0.5 mg mL^{-1} hyaluronidase in HEPES-buffered NCSU-23 medium. Then, the oocytes were washed with 1% PVA in DPBS for 1 min and fixed with absolute ethanol containing 10 μg mL^{-1} Hoechst 33343 for at least 5 min. Then, the oocytes were mounted on glass slides in a drop of 100% glycerol and squashed gently with a coverslip and evaluated fluorescence microscopy. Oocytes at metaphase-II were considered to have matured.

Embryo evaluation and nuclear staining: The embryos were assessed for cleavage on day 2 and for blastocyst development on day 7. The day of activation was day 0. Blastocysts considered viable were washed with 1% PVA in DPBS for 1 min. The nuclear staining were followed as described before.

Intracellular GSH assay: After IVM (42-44 h), the oocytes were stripped of surrounding cumulus cells by repeated pipetting and matured oocytes (defined as oocytes in which the first PB was visualized under a stereomicroscope) were selected for GSH measurement. Intracellular GSH was measured as described by Baker *et al.* (1990) with some modification.

Briefly, M-II oocytes from each group were washed three times in 0.2 M sodium phosphate buffer (Na_2HPO_4, NaH_2PO_4 and 10 mM EDTA-2Na, pH 7.2) and groups of 50-60 oocytes (per sample) in 10 μL sodium phosphate buffer were transferred to 1.7 mL microfuge tubes; 10 μL of 1.25 mM phosphoric acid (final concentration of 0.625 M H_3PO_4) in distilled water was added to each sample.

Tubes containing the samples were frozen at -80°C until analysis. GSH concentrations in the oocytes were determined using a 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) GSH reductase (GSSG) recycling assay. Before the assay, the frozen samples were thawed at room temperature, vortexed, centrifuged and microscopically evaluated to ensure complete lysis of the oocytes. The supernatants were transferred to a 96 well microtiter plate and for each sample, 700 μL of 0.33 mg mL^{-1} NADPH in 0.2 M assay buffer containing 10 mM EDTA (stock buffer, pH 7.2), 100 μL of 6 mM DTNB in the stock buffer and 180 μL of distilled water and 1 U per sample of GSSG (Sigma G3664, 441 U mL^{-1}) were added in a conical tube, mixed and immediately added to the sample.

The plate was immediately placed in a microtiter plate reader and optical density was measured with a 405 nm filter (Emax, Molecular Devices, Sunnyvale, CA, USA). The formation of 5-thio-2 nitrobenzoic acid was monitored every 30 sec for 3 min. Standard curves were prepared for

each assay and GSH content per sample was determined by the standard curve. The GSH concentrations (pM oocyte^{-1}) were calculated by dividing the total concentration per sample by the total number of oocytes present in the sample.

Experimental design: In experiment 1 to standardize the optimal concentration of VEGF, different concentrations (0, 5, 50, 50, 500 ng mL^{-1}) were used in IVM medium to determine the maturation rate.

In experiment 2, COCs were matured under different VEGF concentrations to determine total intracellular GSH. In experiment 3, matured oocytes were activated with an electrical pulse (parthenogenesis) to evaluate developmental competence. Based on the above data, a suitable VEGF concentration was used for IVM.

In experiment 4, developmental competences were compared with those for oocytes that had gone through SCNT but that were not supplemented with VEGF. In experiment 5, degree of cumulus cell expansion was compared with those COCs were cultured with VEGF and those were not cultured with VEGF during IVM. The degree of cumulus cells expansion was evaluated according to Hunter and Moor (1987).

Statistical analysis: The statistical analysis was conducted using SPSS Inc. software (PASW Statistics 17). A one way analysis of variance with Duncan multiple-range test was used to assess maturation rates, total GSH levels and parthenogenesis.

The student's t-test was used in experiments 4 and 5 using GraphPad Prism software. All data are presented as mean±SEM. Differences at $p<0.05$ were considered significant.

RESULTS AND DISCUSSION

Effect of different VEGF concentrations on maturation of oocytes: A total of 1258 COCs were used in experiment 1 to determine the optimum VEGF concentration for *in vitro* oocyte maturation. Maturation rate was not significantly different in the control and treatment groups but tended to be higher in the 5 and 50 ng mL^{-1} treatment groups than the control and 500 ng mL^{-1} groups (Fig. 1). Higher VEGF concentrations had no any effect on oocyte maturation and about 73.75% of oocytes reached the M-II stage which was the same as the control group.

Effect of VEGF on intracellular GSH concentration in M-II oocytes: Total GSH concentration was significantly higher in the 5 and 50 ng mL^{-1} (12.68±0.076 and 12.33±0.53, respectively) VEGF groups compared to the control and 500 ng mL^{-1} (10.19±0.66 and 10.54±0.54, respectively) groups (Fig. 2). No significant difference was observed between the 5 and 50 ng mL^{-1} VEGF groups. Effects of different VEGF concentrations on developmental competence of porcine parthenogenetic embryos.

The blastocyst formation rate was significantly ($p<0.05$) higher in the 5 and 50 ng mL^{-1} (58.99±4.70% and 54.00±1.09, respectively) VEGF groups than in the control (30.15±4.52%) and 500 ng mL^{-1} (34.79±4.01%) groups but there was no significant different between 5 and 50 ng mL^{-1} VEGF treatment group. Total cell number per blastocyst was significantly higher in 5 and 50 ng mL^{-1} VEGF treated group than control and 500 ng mL^{-1} VEGF group (Table 1), similarly there was no significant differences between 5 and 50 ng mL^{-1} VEGF groups. No significant difference in the cleavage rate at day 2 was observed but it tended to be higher in all treatment groups than in the control group (Table 1).

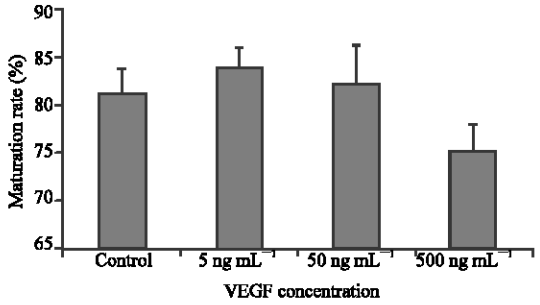

Fig. 1: Effect of different Vascular Endothelial Growth Factor (VEGF) concentrations on maturation rate in porcine cumulus oocyte complexes after a 42 h incubation

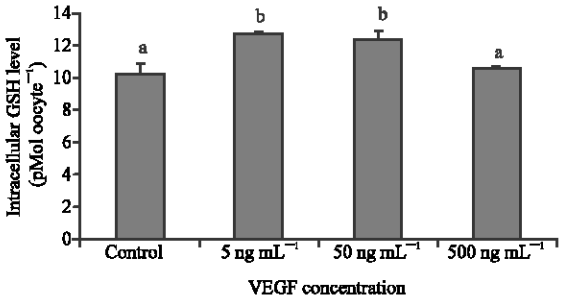

Fig. 2: Intracellular Glutathione (GSH) concentrations of *in vitro* matured porcine oocytes. Oocytes were matured in medium containing different Vascular Endothelial Growth Factor (VEGF) concentrations and compared with the control. Different superscripts represent statistical differences among treatment groups ($p<0.05$)

Effect of VEGF on porcine SCNT embryo developmental ability: As shown in Table 2, embryonic development to the blastocyst stage and total cells number were significantly higher ($p<0.05$) in the VEGF matured oocytes than in the control oocytes. However, the cleavage rate and fusion rate were not significantly different between the two groups.

Effects of VEGF on degree of cumulus cells expansion: The rate of COCs with fully expanded cumulus was significantly ($p<0.05$) higher in 5 ng mL^{-1} VEGF treated group (85.37±0.73%) compared to control (58.89±0.88%). Consequently, the rates of COCs with moderately and slightly expanded cumulus were significantly higher in control group (29.59±1.14, 11.02±0.69%, respectively) compared to 5 ng mL^{-1} VEGF treated group (10.59±0.91, 4.04±1.44%, respectively) during IVM (Table 3).

In vitro embryo production depends on oocyte quality and IVM is an incredibly important technology. There is a deficiency in IVM technology and a need to improve media formulations, especially for immature oocytes (Gilchrist and Thompson, 2007), although live piglets have been produced using IVF, intracytoplasmic sperm injection and SCNT of *in vitro* matured oocytes (Hyun *et al*., 2003; Nakai *et al*., 2003; Suzuki *et al*., 2006). In general, the competence of porcine oocytes/embryos derived from *in vitro* processes is lower than that of their *in vivo* counterparts (Kashiwazaki and Shino, 2001; Lonergan *et al*., 2003).

Incomplete cytoplasmic, maturation is believed to result in abnormal fertilization including polyspermy and asynchronous pronuclear formation (Mattioli *et al*., 1988; Moor *et al*., 1990) which are thought to be the major reasons for poor developmental competence of *in vitro* matured/fertilized embryos (Hunter, 1990). Adding growth factors, cytokines, vitamins or amino acids to *in vitro* culture medium has been studied to improve the quality of *in vitro* produced embryos due to their stimulating and protective effect during culture conditions (Diaz-Cueto and Gerton, 2001; Richter, 2008). The present study was conducted to improve, porcine IVM by supplementing IVM medium with VEGF.

Three different VEGF concentrations were used in the oocyte maturation medium and intracellular GSH concentration of oocytes was considered an oocyte maturation parameter for oocyte developmental competence following parthenogenesis. The results confirmed that supplementing porcine oocyte maturation medium with recombinant human VEGF-165 significantly increased the embryo developmental rate and cell number per blastocyst during parthenogenesis and it was dose depended manner.

This result was similar to that for bovine IVF embryo production (Luo *et al*., 2002a) and suggested that adding exogenous recombinant VEGF to oocyte maturation medium has a beneficial effect on good quality embryo production. In the case of porcine oocyte maturation, VEGF had no effect on PB extraction, a result that differed from bovine oocyte maturation (Luo *et al*., 2002a). This may have been due to the different IVM medium used for bovine oocyte maturation.

SCNT is one of the most time-consuming, technically demanding and labor intensive embryo manipulation methods (Booth *et al*., 2001; Jolliff and Prather, 1997) which is the reason only control and 5 ng mL^{-1} VEGF treatment groups were used for the SCNT in this study.

Table 1: Parthenogenetic developmental ability of porcine oocytes matured under different vascular endothelial growth factor concentrations

Groups	Total oocyte	No. of cleaved (Mean±SEM)*	No. of blastocyst (Mean±SEM)**	Total cell BL^{-1}
Control	109	70 (65.76±10.21)	22 (30.15±4.52)^{a}	56.91±4.78^{a}
5 ng mL^{-1}	125	106 (84.66±3.84)	63 (58.99±4.70)^{b}	83.21±4.89^{b}
50 ng mL^{-1}	121	87 (71.73±3.48)	47 (54.00±1.09)^{b}	78.16±6.15^{b}
500 ng mL^{-1}	102	78 (75.94±3.18)	27 (34.79±4.01)^{a}	55.93±3.89^{a}

Values with different letter superscripts within the same column are significantly different ($p<0.05$), *Percentage of the number of oocytes cultured, **Percentage of the number of oocytes cleaved

Table 2: Developmental competence of porcine somatic cell nuclear transfer embryos matured under 5 ng mL^{-1} Vascular Endothelial Growth Factor (VEGF)

Groups	Reconstructed oocyte	No. of fused (Mean±SEM)	No. of cleaved (Mean±SEM)*	Blastocyst (Mean±SEM)**	Cell BL^{-1}
Control	322	284 (88.00±1.64)	167 (58.67±2.84)	13 (7.95±1.44)^{a}	48.09±5.36^{a}
VEGF	294	247 (84.12±1.99)	158 (64.01±2.69)	23 (14.54±1.42)^{b}	67.83±6.56^{b}

Values with different letter superscripts within the same column are significantly different ($p<0.05$), *Percentage of the number of oocytes fused, ** Percentage of the number of oocytes cleaved

Table 3: Effects of Vascular Endothelial Growth Factor (VEGF) (5 ng mL^{-1}) on porcine cumulus cell expansion

Groups	Total oocyte cultured	Cumulus complexes	Fully expanded	Moderately expanded	Slightly expanded
Control	382	-	225 (58.89±0.88)^{a}	113 (29.59±1.14)^{c}	44 (11.02±0.69)^{e}
VEGF	341	-	291 (85.37±0.73)^{b}	36 (10.59±0.91)^{d}	14 (4.04±1.44)^{f}

Values with different letter superscripts within the same column are significantly different ($p<0.05$)

However, significantly higher embryo developmental rates and cell number per blastocyst were observed during SCNT after adding VEGF to the porcine IVM medium. In this study, we observed that intracellular GSH was also significantly increased in 5 and 50 ng mL^{-1} VEGF treated groups compared to control and 500 ng mL^{-1} VEGF treated group.

GSH is the major non-protein sulfhydryl component in mammalian cells and plays an important role in protecting the cell from oxidative stress and toxic Reactive Oxygen Species (ROS) activity (Luberda, 2005). Synthesis of GSH during oocyte maturation has been reported in the mouse (Calvin *et al*., 1986), hamster (Noda *et al*., 1991), pig (Yoshida *et al*., 1993) and cow (De Matos *et al*., 1995).

GSH content increases during development and oocyte maturation in the ovary as the oocyte approaches ovulation. It has been suggested that intracellular GSH concentrations in porcine oocytes at the end stage of IVM reflect the degree of cytoplasmic maturation (Funahashi *et al*., 1994). This result indicated that intracellular GSH level is important for *in vitro* porcine embryo production and it was influenced by dose dependent. Several studies suggested that intracellular GSH may play an important role in many biological process including DNA and protein synthesis, cellular protection during oxidative stress and cell proliferation during embryonic events (Lafleur *et al*., 1994; Yu, 1994). However, until now it was unclear how VEGF increases intracellular GSH concentration during oocyte maturation. Intracellular GSH synthesis was believed to be regulated by cumulus cells (Luo *et al*., 2002b; Maedomari *et al*., 2007).

The results showed that adding VEGF to IVM medium enhanced cumulus cell expansion significantly compared with a control group. Maximal *in vitro* cumulus expansion improves the capacity of oocytes for subsequent embryo development to the blastocyst stage and the synthesis of intracellular GSH content is dependent on optimal cumulus expansion (Furnus *et al*., 1998).

ROS generation is a problem during *in vitro* embryo manipulation because ROS react with extremely high rate constants with sugars, amino acids, phospholipids, nucleotides and organic acids which damage cell membranes and play a role in apoptosis. The deleterious effects of ROS on embryos are well reviewed by Guerin *et al*. (2001).

Electrical activation increases ROS production in porcine embryos and exogenous GSH minimizes these adverse effects (Koo *et al*., 2008). In the experiments, electrical pulses were used to either activate or fuse and activate the cells. The increased endogenous GSH may have helped to minimize the deleterious effects of ROS on embryos. Male Pro-Nuclear (MPN) formation and cytoplasmic GSH concentrations are correlated during porcine embryo development (Maedomari *et al*., 2007). Elevated intracellular GSH during oocyte IVM results in enhanced sperm nuclear decondensation and MPN formation during fertilization in mammals (Funahashi *et al*., 1995; Perreault *et al*., 1988; Yoshida, 1993). After activation, embryonic development is related to pronuclei formation during parthenogenesis and SCNT. Although, it was not demonstrated, elevated intracellular GSH could help to promote pronuclear formation after porcine oocyte activation.

CONCLUSION

In this study, VEGF supplementation during IVM of porcine oocytes could help cytoplasmic maturation by increasing intracellular GSH concentrations and also improve developmental competence in parthenogenetic and SCNT embryos. The results hypothesized that increasing GSH concentrations might be involved to stimulate developmental related gene expression in young embryos.

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