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Supplementation of Vascular Endothelial Growth Factor during *in vitro* Maturation of Porcine Cumulus Oocyte Complexes and Subsequent Developmental Competence after Parthenogenesis and *in vitro* Fertilization

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Abstract**—**In mammalian reproductive tract, the oviduct secretes huge number of growth factors and cytokines that create an optimal micro-environment for the initial stages of preimplantation embryos. Secretion of these growth factors is stage-specific. Among them, VEGF is a potent mitogen for vascular endothelium and stimulates vascular permeability. Apart from angiogenesis, VEGF in the oviduct may be involved in regulating the oocyte maturation and subsequent developmental process during embryo production *in vitro*. In experiment 1, to evaluate the effect of VEGF during IVM of porcine COC and subsequent developmental ability after PA and SCNT. The results from these experiments indicated that maturation rates among the different VEGF concentrations were not significant different. In experiment 2, total intracellular GSH concentrations of oocytes matured with VEGF (5-50 ng/ml) were increased significantly compared to a control and VEGF group (500 ng/ml). In experiment 3, the blastocyst formation rates and total cell number per blastocyst after parthenogenesis of oocytes matured with VEGF (5-50 ng/ml) were increased significantly compared to a control and VEGF group (500 ng/ml). Similarly, in experiment 4, the blastocyst formation rate and total cell number per blastocyst after SCNT and IVF of oocytes matured with VEGF (5 ng/ml) were significantly higher than that of oocytes matured without VEGF group. In experiment 5, at 10 hour after the onset of IVF, pronuclear formation rate was evaluated. Monospermy was significantly higher in VEGF-matured oocytes than in the control, and polyspermy and sperm penetration per oocyte were significantly higher in the control group than in the VEGF-matured oocytes. Supplementation with VEGF during IVM significantly improved male pronuclear formation as compared with the control. In experiment 6, type III cortical granule distribution in oocytes was more common in VEGF-matured oocytes than in the control. In conclusion, the present study suggested that supplementation of VEGF during IVM may enhance the developmental potential of porcine *in vitro* embryos through increase of the intracellular GSH level, higher MPN formation and increased fertilization rate as a consequence of an improved cytoplasmic maturation.

Keywords**—**angiogenesis, GSH, monospermy, VEGF

I. Introduction

GAMETE maturation is a crucial process for the generation of a healthy, genetically normal conceptus. Additionally, supplementation of growth factors and cytokines in traditional IVM or *in vitro* culture (IVC) medium affects oocyte or embryo quality. For successful implantation, good-quality preimplantation embryos are essential for a full-term birth. Addition of growth factors to traditional IVC medium has been shown to reduce degeneration, fragmentation, and apoptosis, and increase blastocyst formation, cell number, hatching rate, implantation rate, and pregnancy or birth rates in a variety of animal embryos [1]. Exogenous growth factors affect nuclear and cytoplasmic maturation, and induce genes that are essential for successful implantation. Cytoplasmic maturation involves the accumulation of messenger RNA (mRNA), intracellular glutathione (GSH), proteins, substrates, and nutrients that are required to achieve oocyte developmental competences that foster embryonic developmental competence [2]. Vascular endothelial growth factor (VEGF) is potent mitogen specific for vascular endothelial cells and acts through its corresponding receptors. Addition of VEGF to bovine IVM medium improved developmental competence after *in vitro* fertilization (IVF) [3]. The exact mechanism remains unclear. Along with proliferative effects on endothelial cells, VEGF acts as a cytoprotective agent, protecting the cell from apoptosis [4]. The present study was undertaken to characterize the specific roles of VEGF during both *in vitro* porcine oocyte maturation and porcine embryonic development. This goal was approached by analyzing the developmental competences were evaluated by adding VEGF to traditional porcine IVM medium through IVF, parthenogenesis, and somatic cell nuclear transfer (SCNT).

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II. Materials and Methods

A. Ovary Collection, Recovery and In Vitro Oocyte Maturation

Ovaries of pre-pubertal gilts were collected from a commercial abattoir. The follicular contents were aspirated from 3- to 7-mm superficial antral follicles with a 10 ml disposable syringe and 20-gauge needle. Cumulus-oocyte complexes (COC) with at least three layers of compact cumulus cells and with homogenous cytoplasm were selected and a group of 50-60 COCs were cultured in tissue culture medium-199 (M-199) (Invtrogen, Grand Island, NY) supplemented with 0.6 mM cysteine, 0.91 mM pyruvate, 15 ng/ml epidermal growth factor, 75 µg/ml kanamycin, 1 µg/ml insulin and 10% porcine follicular fluid in each well of four-

well multi dish (Nunc, Roskilde, Denmark). The COCs were then statically cultured at 39°C in a humidified atmosphere containing 5% CO2 with 10 IU/ml eCG (Intervet International BV, Boxmeer, Holland) and 10 IU/ml hCG (Intervet International BV). After 20–22 h of maturation with hormones, the oocytes were washed two times in fresh maturation medium before being cultured in hormone-free medium for an additional 18 h for SCNT and 22 h for PA. The pFF was and stored at -20ºC until use.

B. In Vitro Fertilization

After the completion of culture of oocytes for IVM, cumulus cells were removed with 0.1% hyaluronidase in TL-HEPES-PVA and washed three times with mTBM containing 1 mM caffeine and 0.1% BSA (Sigma). After washing, 10-15 oocytes were placed in 40 µl mTBM drops that had been covered with warm mineral oil. For IVF, 0.5 ml liquid semen was washed two times by centrifugation at 1900 x g for 2 min in DPBS (Gibco, Grand Island, NY) supplemented with 0.1% BSA. Thereafter, the sperm pellet was re-suspended with IVF medium and appropriate sperm concentration was made and subsequently 5 µL of sperm suspension was added in IVF drop that contained MII oocytes (final concentration 2.5 X 10^5 sperm/ml). Oocytes were co-incubated with spermatozoa at 39^oC in an atmosphere of 5% CO_2 in air for 20 min. After that, the oocytes were gently washed with TL-HEPES-PVA and transferred to a fresh 50 µL droplet of the same medium without spermatozoa and the culture continued until 6 h after insemination. Before insemination, motility was assessed by placing a drop of sperm suspension on a warm glass slide and examining it subjectively at X 100 magnification. After 6 h, gametes were removed from the fertilization drops, washed three or four times in TL-HEPES-PVA Medium and cultured in 25 µl microdrops (~10 gametes/drop) of porcine zygotic medium-3 (PZM-3) covered with worm mineral oil and incubated at 39°C for 168 hours under 5% O_2 , 5% CO_2 , 90% N_2. Cleavage and blastocyst formation were evaluated under a stereomicroscope at 48 and 168 hours post-insemination, respectively. The day of insemination was considered as day 0.

C. Donor Cells Preparation, SCNT and Parthenogenesis

Porcine ear skin fibroblasts from adult female pigs were seeded in to four-well plates and were grown in Dulbecco's modified Eagle medium (DMEM) with 1 mM sodium pyruvate, 1% (v/v) non-essential amino acids (Invitrogen, USA), and 10 µg/ml penicillin–streptomycin solution, which was supplemented with 10% (v/v) fetal bovine serum from a single batch until a complete monolayer of cells had formed. The donor cells were synchronized at the G0/G1 stage of the cell cycle by contact inhibition for 3–4 days. The cells of the same passage were used in each replicate for the various treatments. The individual cells were retrieved from the monolayer by trypsinization for ~1 min and subsequently used for SCNT.

After 40 h of IVM, cumulus-cell-free oocytes were incubated for 2 min in manipulation medium (calcium free TLH-BSA) containing 5 µg/ml Hoechst 33343 and 7.5 µg/ml cytoclasin B (Sigma-Aldrich Co). Following incubation, the oocytes were transferred into a drop of manipulation medium containing 7.5 µg/ml cytoclasin B and were overlaid with warm mineral oil. The zona pellucida was partially dissected with a fine glass needle near the first polar body (PB). The first PB and adjacent cytoplasm (~10%), presumably containing the metaphase-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 injecting pipette, a 12–15-µm trypsinized fetal fibroblast with a smooth surface was transferred into the periviteline space through the same slit of an enucleated oocyte. The reconstructed couplets were equilibrated with 0.26 M mannitol containing 0.5 mM HEPES, 0.001 mM CaCl2, and 0.05 mM MgSO4 for 2–3 min and transferred to a 1 mm fusion chamber containing overlaid with same mannitol solution. Membrane fusion and activation were done according to describe elsewhere. Activated oocytes were washed and cultured in PZM-3 medium supplemented with 3 mg/ml fatty-acid free BSA and placed in humidified incubator at 39°C under 5% CO2.

For PA, the MII oocytes at 42 h of IVM were activated using a pulse sequence identical to that used to activate SCNT oocytes. The culture procedures of PA embryos were similar to SCNT embryos. Cleavage and blastocyst formation were evaluated at 48 and 168 h post activation, respectively, with the day of SCNT or PA designated Day 0.

D. Intracellular GSH Assay

After IVM (42–44 hours), the surrounding cumulus cells were removed by repeated pipetting, and matured oocytes were selected for GSH measurement. M-II oocytes from each group were washed three times in 0.2 M sodium phosphate buffer (Na_2HPO4, 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 nicotinamide adenine dinucleotide phosphate (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) 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) were calculated by dividing the total concentration per sample by the total number of oocytes present in the sample.

E. Evaluating the Number of Sperm Bound to the Plasma Membrane and Pronuclear Formation Test

Cell staining to detect sperm bound to the plasma membrane and pronuclear formation was performed according to Koo *et al.* (2005) with some modifications. Briefly, at 10 hours after insemination, the ZP of oocytes was dissolved with 0.5% pronase. The zona-free embryos were washed in TL-HEPES medium containing 0.1% formaldehyde and 0.01% PVA for 1 min and fixed in 1% formaldehyde and 0.01% PVA in DPBS for 10 min at room temperature. The fixed embryos were placed in a drop of mounting medium [25% (*v/v*) glycerol in DPBS containing 2.5 mg/ml sodium azide and 2.5 µg/ml Hoechst stain] on a slide, and a cover slide was placed over the embryos. The number of spermatozoa penetrating the ZP, the presence of polyspermy, and MPN formation were examined under a fluorescence microscope.

F. CGs Distribution Assessment

Staining to determine the CG distribution was performed with fluorescein isothiocyanate-labeled peanut agglutinin (FITC-PNA) according to describe elsewhere with a few modifications. Briefly, at the end of the maturation period, all oocytes were denuded by gentle pipetting and washed in DPBS containing 0.3% BSA (Sigma). The M-II oocytes were selected, the ZP was removed by treatment with 0.2% pronase in DPBS, and the oocytes were washed twice for 5 min in DPBS containing 0.3% BSA. The zona-free oocytes were fixed with 3.7% (w/v) paraformaldehyde in DPBS for 30 min at room temperature and washed three times for 5 min in a blocking solution of 0.3% BSA and 100 mM glycine (Wako Pure Chemical, Osaka, Japan) in DPBS. The fixed oocytes were treated with 0.1% (*v/v*) Triton X-100 in DPBS for 5 min, washed twice for 5 min in blocking solution, and incubated with FITC-PNA (10 µg/ml in DPBS) for 30 min in the dark. After staining, the oocytes were washed three times in DPBS containing 0.3% BSA and 0.01% Triton X-100 and mounted on non-fluorescent glass slides with a cover slip secured by nail polish. The slides were evaluated under a laser scanning confocal microscope (Bio-Rad MRC 600) equipped with a krypto-argon ion laser for the excitation of fluorescein-labeled CGs. The images were recorded digitally and archived on an erasable magnetic optical disk. The CG distribution patterns were classified into three categories (see text).

G. Embryo Evaluation and Nuclear Staining

Blastocysts considered viable were washed with 1% PVA in Dulbecco's phosphate buffered saline (DPBS) for 1 min and then fixed with 100% ethanol containing 10 µg/ml Hoechst for 5 min. Then, the blastocysts were mounted on glass slides in a drop of 100% glycerol and squashed gently with a coverslip. The nuclei were counted using fluorescence microscopy.

H. Experimental Design

In experiment 1, to standardize the optimal concentration of VEGF, different concentrations (0, 5, 50, 50, 500 ng/ml) 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 and IVF but that were not supplemented with VEGF. In experiment 5, sperm penetration and pronuclear formation rate was compared between VEGF matured and control group of oocytes. In experiment 6, cortical granules distribution pattern was compared between VEGF-matured and without VEGF-matured oocytes.

III. 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, 5 and 6 using GraphPad Prism software. All data are presented as mean±SEM. Differences at $p<0.05$ were considered significant.

IV. Results

A. 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 treatment groups than the control and 500 ng/ml 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.

B. Effect of VEGF on Intracellular GSH Concentration in M-II Oocytes

Total GSH concentration was significantly higher in the 5 and 50 ng/ml (12.68 ± 0.076 and 12.33 ± 0.53, respectively) VEGF groups compared to the control and 500 ng/ml (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 VEGF groups.

C. 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 (58.99 ± 4.70% and 54.00 ± 1.09,

respectively) VEGF groups than in the control (30.15 ± 4.52%) and 500 ng/ml (34.79 ± 4.01%) groups but there was no significant different between 5 and 50 ng/ml VEGF treatment group. Total cell number per blastocyst was significantly higher in 5 and 50 ng/ml VEGF treated group than control and 500 ng/ml VEGF group (Table I), similarly there was no significant differences between 5 and 50 ng/ml 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 I).

TABLE I
PARTHENOGENETIC DEVELOPMENTAL ABILITY OF PORCINE OOCYTES MATURED UNDER DIFFERENT VASCULAR ENDOTHELIAL GROWTH FACTOR CONCENTRATIONS

Group	Total oocyte	No. of Cleaved (%)*	No. of Blastocyst (%)**	Total cell/BL
Control	109	70 (65.76 ± 10.21)	22 (30.15 ± 4.52)	56.91 ± 4.78
5 ng/ml	125	106 (84.66 ± 3.84)	63 (58.99 ± 4.70)[a]	83.21 ± 4.89[a]
50 ng/ml	121	87 (71.73 ± 3.48)	47 (54.00 ± 1.09)[a]	78.16 ± 6.15[a]
500 ng/ml	102	78 (75.94 ± 3.18)	27 (34.79 ± 4.01)	55.93 ± 3.89

*Percentage of the number of oocytes cultured
** Percentage of the number of oocytes cleaved
Data were presented as mean ± SEM of each treatment.
[a] $p<0.05$ vs. control and 500 ng/ml group within the same column

D. Effect of VEGF on porcine SCNT and IVF embryo developmental ability

As shown in Table II, embryonic development to the blastocyst stage and total cells number were significantly higher ($p<0.05$) in SCNT and IVF embryos in the VEGF-matured oocytes than in the control oocytes. However, the cleavage rate was not significantly different between the two groups.

TABLE II
EFFECTS OF VEGF (5 ng/ml) DURING *IN VITRO* MATURATION OF PORCINE COCS AND DEVELOPMENTAL POTENTIAL AFTER IVF AND SCNT

Type of embryos	Treatment group	Total number of zygote examined	Cleavage rate (%)	Blastocyst rate (%)*	Total cell number
IVF	Control	126	77 (61.2 ± 2.0)	25 (32.5 ± 3.4)[a]	64.1 ± 5.6[a]
	VEGF	148	100 (68.3 ± 3.9)	45 (46.7 ± 3.1)[b]	82.8 ± 6.7[b]
SCNT	Control	284	167 (58.67 ± 2.84)	13 (7.95 ± 1.44)[a']	48.09 ± 5.36[a']
	VEGF	247	158 (64.01 ± 2.69)	23 (14.54 ± 1.42)[b']	67.83 ± 6.56[b']

*Percentage of cleaved embryos
Data were presented as mean ± SEM of each treatment
[a,b and a',b'] $p<0.05$ vs. control group within same column and within same type of embryos

E. Effects of VEGF Treatment During Porcine COC Maturation on Sperm Penetration and Pronuclear Formation After IVF

The MPN formation was significantly ($p<0.05$) higher in COCs that matured in the presence of VEGF (5 ng/ml) compared with the control COCs without VEGF (91.1 ± 1.9% vs. 74.4 ± 3.8, respectively). Monospermy was significantly higher in VEGF-matured oocytes (47.2 ± 4.3%) than in the control (20.0 ± 2.4%), and polyspermy and sperm penetration per oocyte were significantly higher in the control group (54.4 ± 3.8% and 2.3 ± 0.1, respectively) than in the VEGF-matured oocytes (43.9 ± 3.6% and 1.8 ± 0.1, respectively). There was no significant difference in the sperm penetration rate between the control and VEGF-treated groups. Owing to a higher rate of polyspermy in the control oocytes, the polypronuclear oocyte formation rate was significantly higher in the control oocytes than in the VEGF-matured oocytes (Table III).

TABLE III
EFFECT OF VEGF SUPPLEMENTATION (5 ng/ml) DURING PORCINE COCS MATURATION ON SPERM PENETRATION AND PRONUCLEAR FORMATION AFTER IVF

Parameter	Control	VEGF	Significance
No. of oocytes examined	145	154	
Penetration (%)[a]	137 (94.7 ± 2.9)	141 (91.0 ± 3.0)	NS
MPN formation (%)[b]	102 (74.4 ± 3.8)	128 (91.1 ± 1.9)	S
Monospermy (%)[c]	27 (20.0 ± 2.4)	65 (47.2 ± 4.3)	S
Polyspermy (%)[d]	75 (54.4 ± 3.8)	63 (43.9 ± 3.6)	S
3PN	30 (22.5 ± 2.9)	24 (16.2 ± 2.9)	NS
4PN	24 (16.0 ± 3.2)	31 (22.1 ± 2.5)	NS
5PN	21 (16.0 ± 4.6)	8 (5.7 ± 1.9)	S
Sperm/penetrated egg	2.3 ± 0.1	1.8 ± 0.1	S

[a]Percentage of the number of oocytes examined
[b-d]Percentage of the number of oocytes penetrated
Data are given as mean ± SEM from seven replicated of each treatment

F. Effects of VEGF on CG Distribution in Porcine M-II Oocytes

After 42 hours, type III CG distribution was significantly ($p<0.001$) higher in the VEGF-supplemented group than in the control group, and type II CG distribution was significantly higher ($p<0.001$) in the control group than in the VEGF-supplemented group (Table IV, Fig. 1).

V. Discussion

It has been suggested that intracellular GSH concentrations in porcine oocytes at the end stage of IVM reflect the degree of cytoplasmic maturation [5]. 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 [6]. In this study is was observed that, intracellular GSH was also significantly increased in 5 and 50 ng/ml VEGF treated groups compared to control and 500 ng/ml VEGF treated group. GSH content increases during development and oocyte maturation in the

ovary as the oocyte approaches ovulation. However, until now, it was unclear how VEGF increases intracellular GSH concentration during oocyte maturation. Electrical activation increases ROS production in porcine embryos, and exogenous GSH minimizes these adverse effects [7]. In this 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.

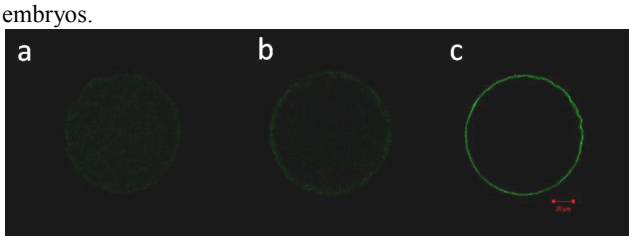

Fig. 1 Confocal images of zona-free pig oocytes stained with FITC-PNA for demonstrating type of CGs distribution in porcine MII oocytes. Type I, complete distribution of CGs throughout the entire ooplasm (a), Type II, partially localized of CGs near to ooplasmic membrane (b), Type III, distribution of CGs in the cortex of cytoplasm aligning with ooplasmic membrane (c). Scale bar=20 µm

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 [3] and suggested that adding exogenous recombinant VEGF to oocyte maturation medium has a beneficial effect on good-quality embryo production.

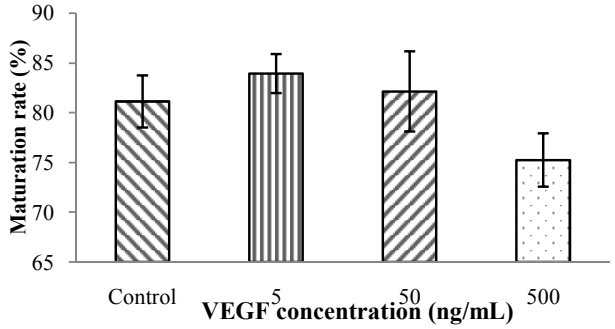

Fig. 2 Effect of different concentrations of VEGF on porcine oocytes maturation rate after 42 hour of incubation (p>0.05). Data were presented as mean ± SEM of each treatment

In the case of porcine oocyte maturation, VEGF had no effect on PB extraction, a result that differed from bovine oocyte maturation [3]. 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 [8], [9], which is the reason only control and 5 ng/ml VEGF treatment groups were used for the SCNT in this study. However, significantly higher embryo developmental rates and cell number per blastocyst were observed during SCNT after adding VEGF to the porcine IVM medium. We also showed that VEGF supplementation during IVM of porcine oocytes significantly increased embryonic developmental potential and the total cell number per blastocyst during IVF compared with the control group. The results of the present studies demonstrate that VEGF supplementation during IVM can improve the developmental potential of IVF, PA, and nuclear transfer embryos. Thus, it may be that the beneficial effects of VEGF are mediated by direct or indirect effects on GSH synthesis in COCs.

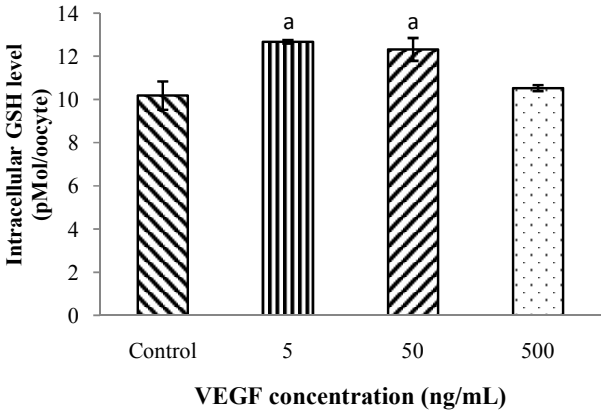

Fig. 3 Intracellular glutathione (GSH) concentrations of *in vitro* matured porcine oocytes. Oocytes were matured in medium containing different vascular endothelial growth factor (VEGF) concentrations and compared with the control. Data represent the mean ± SEM of each treatment. [a] p<0.05 vs. control and 500 ng/ml

Polyspermic penetration occurs more frequently in pigs than any other species and leads to unresolved problems with respect to IVF [10]. Cytoplasmic maturation is an important factor for preventing polyspermy in mammals. In mammalian *in vitro* matured oocytes, intracellular GSH plays an important role in MPN formation after fertilization [11], [12]. In the present study, polyspermy and sperm penetration per oocyte were significantly reduced in VEGF-matured oocytes as compared with the controls. Monospermy and the MPN formation rate were significantly higher in VEGF-matured oocytes than in the control. The synthesis of intracellular GSH during oocyte maturation appears to be a prerequisite for initiating sperm chromatin decondensation prior to MPN formation in oocytes of mouse [13], hamster [14], and pig [5], [15]. Furthermore, high intracellular GSH also appears to be essential for overcoming the *in vitro* developmental arrest in mice and rats embryos [16]. In general, oocyte cytoplasmic quality is a major determinant of embryonic developmental potential during *in vitro* embryo production. Cytoplasmic organelle redistribution and migration occur during meiosis, and the CG density and cortical localization in matured oocytes are important markers for cytoplasmic maturation [17].

TABLE IV
CG DISTRIBUTION PATTERN OF PORCINE MII OOCYTES AFTER 42 HOURS MATURED WITH VEGF (5 ng/ml).

Treatment group	Total number of M-II oocytes	CG distribution pattern* (%)		
		Type I	Type II	Type III
Control	56	4 (7.1 ± 1.5)	23 (40.8 ± 2.2)	29 (52.1 ± 2.1)
VEGF	59	2 (3.4 ± 1.7)	11 (18.6 ± 1.4)[a]	46(78.0 ± 1.5)[a]

* Please see the text for description of CG distribution pattern.
Data were presented as mean ± SEM of each treatment
[a] $p<0.001$ vs. control group within same column(Student t-test at $p<0.001$)

In the present study, about 47.9% of control oocytes exhibited significantly delayed migration and dispersal of CGs (type I and II patterns) as compared with the VEGF-matured oocytes (22.0%), whereas about 78% of the VEGF-matured oocytes displayed type III CG distribution, compared with 52.1% of controls oocytes. These results indicate that VEGF supplementation during IVM improves cytoplasmic maturation. Type III CG distribution reflects complete cytoplasmic maturation and these oocytes are competent to develop to blastocyst stage after IVF. In our study, the blastocyst rate was 46.7% in VEGF-matured oocytes, which is similar to the rate in bovine oocytes [3]. The MPN formation rate and monospermy were significantly improved, and consequently polyspermy and sperm penetration per oocyte were significantly reduced, in VEGF-matured oocytes as compared with the control oocytes after IVF. This may be attributable to complete translocation of CGs to the cortex owing to the functional effects of VEGF supplementation during IVM of porcine COCs.

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