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Supplementation with vascular endothelial growth factor during *in vitro* maturation of porcine cumulus oocyte complexes and subsequent developmental competence after *in vitro* fertilization

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Abstract

The aim of the present study was to investigate whether the effects of vascular endothelial growth factor (VEGF) on porcine cumulus oocyte complexes (COCs) and subsequent blastocyst formation following *in vitro* fertilization are attributable to improved fertilization and cytoplasmic maturation. Porcine COCs were cultured for 42 h in TCM199 medium with 5 ng/mL human recombinant VEGF, and the resultant metaphase II oocytes were fertilized *in vitro*. COCs without VEGF supplementation served controls. Supplementation with VEGF during *in vitro* maturation (IVM) significantly ($P < 0.05$) improved the blastocyst formation rate and total cell number ($46.7 \pm 3.1\%$ and 82.8 ± 6.7, respectively) compared with controls ($32.5 \pm 3.4\%$ and 64.1 ± 5.6, respectively). On day 2, the percentage of four-cell stage embryos was significantly higher in the VEGF-matured group ($49.1 \pm 2.7\%$) than in the control ($33.1 \pm 5.8\%$), and the percentage of two-cell stage embryos was significantly higher in the control group ($10.4 \pm 1.4\%$) than in the VEGF-matured group ($6.6 \pm 0.9\%$). At 10 h after the onset of *in vitro* fertilization (IVF), oocytes with two pronuclei were considered as monospermically or normally fertilized, and oocytes with more than two pronuclei were considered as polyspermically fertilized. Monospermy was significantly higher in VEGF-matured oocytes ($47.2 \pm 4.3\%$) than in the control ($20.0 \pm 2.4\%$), and polyspermy and sperm penetration per oocyte were significantly higher in the control group ($54.4 \pm 3.8\%$ and 2.3 ± 0.1, respectively) than in the VEGF-matured oocytes ($43.9 \pm 3.6\%$ and 1.8 ± 0.1, respectively). Supplementation with VEGF during IVM significantly ($P < 0.05$) improved male pronuclear formation as compared with the control (91.1 ± 1.9 vs $74.4 \pm 3.8\%$). Type III cortical granule distribution in oocytes was more common in VEGF-matured oocytes (78.0%) than in the control (52.1%). These results suggest that VEGF supplementation during IVM enhanced the developmental potential of porcine IVF embryos through higher male pronuclear formation and higher monospermic fertilization rates as a consequence of improved cytoplasmic maturation.
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Keywords: VEGF; Polyspermy; MPN; Cortical granule

1. Introduction

Polyspermic penetration is a major problem encountered with the in vitro fertilization (IVF) of porcine oocytes which reduces the efficiency of in vitro embryo production (IVP) and still it is an unresolved problems in the porcine IVF programme [1,2]. For in vitro systems, the rate often exceeds 50% [3,4]. It can be induced in vivo when abnormal conditions are imposed experimentally upon oocytes, including the postovulatory ageing of oocytes [5], injection of progesterone beneath the serosal layer of the fallopian tubes or sys-

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temically, and introduction of an excessive number of spermatozoa into the fallopian tubes shortly before ovulation. The incidence of polyspermic penetration observed for *in vivo* porcine eggs us substantially higher (30-40%) than the other species [6]. In mice, hardening of the zona pellucida (ZP), which is associated with the exocitosis of cortical granules (CG) in the cortical reaction and cleavage of ZP2 protein, is thought to be a major component of the protective mechanisms against polyspermy [7].

Polyspermy causes aberrant development and the death of early embryos [6]. It is associated with insufficient cytoplasmic and nuclear maturation, which leads to low fertilization and blastocyst rates. The success of nuclear and cytoplasmic maturation of oocytes is crucial for efficient IVM/IVF embryo production. During cytoplasmic maturation, the cell becomes prepared for fertilization, activation, and embryo development. In various model organisms, this includes the ability of competent female germ cells to fuse with sperm, decondensation of sperm chromatin, formation of pronuclei, and prevention of polyspermy.

Vascular endothelial growth factor (VEGF) is a homodimer with a subunit molecular mass of 23 kDa [8]. It is a potent mitogen for micro- and macrovascular endothelial cells [9,10]. In the female reproductive system, VEGF is involved in many processes such as ovulation, periodic changes of the endometrium, embryo implantation, and development [11]. There is evidence that adding exogenous VEGF during *in vitro* maturation of bovine oocytes significantly improves the rates of cleavage and zygote development [12]. This effect occurs synergistically with follicle-stimulating hormone (FSH) [13] or cumulus cell expansion [14]. Whether improved cleavage rates and embryonic development of porcine oocytes during IVM/IVF are attributable to enhanced fertilization is not clear. However, we previously have shown that VEGF supplementation of porcine IVM medium influences the developmental potential of parthenogenetic and somatic cell nuclear transfer embryos by increasing intracellular glutathione (GSH) levels [15]. Optimal cytoplasmic maturation may be involved in the prevention of polyspermy during *in vitro* embryo production and for increased male pronuclear formation after fertilization. The present study was designed to investigate the effects of VEGF on *in vitro* maturation of porcine oocytes and subsequent developmental competence after *in vitro* fertilization.

2. Material and methods

2.1. 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 at 30 to 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. COCs with at least three layers of compact cumulus cells and with homogenous cytoplasm were selected for IVM. The selected 40–45 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 epidermal growth factor, 0.5 IU/mL porcine luteinizing hormone, 0.5 IU/mL porcine follicle stimulating hormone, 10% (v/v) porcine follicular fluid (pFF), 75 μg/mL penicillin-G, and 50 μg/mL streptomycin. The pFF was aspirated from 3–7 mm follicles of prepubertal gilt ovaries and were prepared according to Hyun *et al.* [16] and stored at –20 °C until use. The COCs were then statically cultured at 39 °C in a humidified atmosphere containing 5% CO_2 with 10IU/mL 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 20 h.

2.2. 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 $\times$ g for 2 min in Dulbecco's PBS (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 $\times$ 10^5 sperm/mL). Oocytes were co-incubated with spermatozoa at 39 °C 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 × 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) [17] covered with worm mineral oil and incubated at 39 °C for 168 hr under 5% O_2, 5% CO_2, 90% N_2. Cleavage and blastocyst formation were evaluated under a stereomicroscope at 48 and 168 h post-insemination, respectively. The day of insemination was considered as day 0.

2.3. Evaluating the number of sperm bound to the plasma membrane and pronuclear formation test

Cell staining to detect sperm penetration and pronuclear formation was performed according to Koo et al [18] with some modifications. Briefly, at 10 h 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 PBS for 10 min at room temperature. The fixed embryos were placed in a drop of mounting medium [25% (v/v) glycerol in PBS 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 male pronuclear formation (MPN) were examined under a fluorescence microscope.

2.4. Embryo evaluation and nuclear staining

Blastocysts considered viable were washed with 1% PVA in DPBS for 1 min and then fixed with 100% ethanol containing 10 μg/mL Hoechst 33258 for at least 5 min at room temperature. Then the blastocyst were mounted on glass slides in a drop of 100% glycerol and squashed gently with a cover slip. The stained nuclei were counted using fluorescence microscopy.

2.5. Cortical granules distribution assessment

Staining to determine the CG distribution was performed with fluorescein isothiocyanate-labeled peanut agglutinin (FITC-PNA) according to Yoshida et al [19] with a few modifications. Briefly, at the end of the maturation period, all oocytes were denuded by gentle pipetting and washed in PBS containing 0.3% BSA (Sigma). The MII oocytes were selected, the ZP was removed by treatment with 0.2% pronase in PBS, and the oocytes were washed twice for 5 min in PBS containing 0.3% BSA. The zona-free oocytes were fixed with 3.7% (w/v) paraformaldehyde in PBS 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 PBS. The fixed oocytes were treated with 0.1% (v/v) Triton X-100 in PBS for 5 min, washed twice for 5 min in blocking solution, and incubated with FITC-PNA (10 μg/mL in PBS) for 30 min in the dark. After staining, the oocytes were washed three times in PBS 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 according to Izadyar et al [20].

2.6. Statistical analysis

The statistical analysis was conducted using student's *t*-test using GraphPad Prism software. All data are presented as mean±SEM. Differences at $P < 0.05$ were considered significant.

3. Results

3.1. Effects of VEGF on the developmental potential of porcine IVF embryos

As shown in Table 1, the embryonic developmental potential to the blastocyst stage and the total cell number per blastocyst were significantly higher ($P < 0.05$) in VEGF-matured oocytes as compared with control oocytes without VEGF. On day 2, the total cleavage rate did not differ significantly between the two groups; the percentage of four-cell stage embryos was significantly higher in the VEGF-matured group (49.1 ±

Table 1
Effects of VEGF (5 ng/mL) during *in vitro* maturation of porcine COCs and developmental potential after IVF.

Treatment group	Total oocyte examined	Cleavage rate (%)	Blastocyst rate (%)*	Total cell number
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]

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

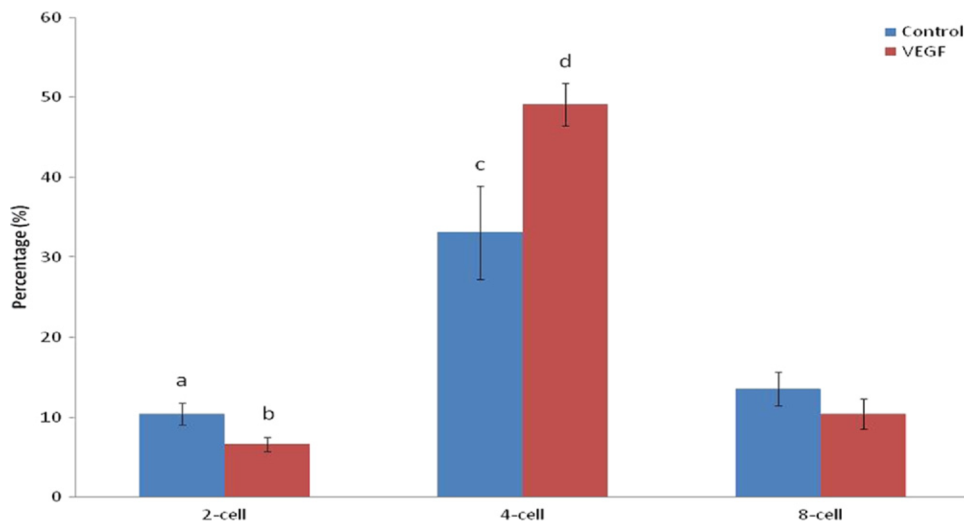

Fig. 1. Cleavage pattern at day 2 of porcine *in vitro* fertilized embryo after maturation of COCs with VEGF (5 ng/mL). Data represented as mean ± SEM of individual experiments. The letters (a, b, c, d) above the columns indicate statistical significance at $P < 0.05$.

2.7%) than in the control (33.1 ± 5.8%); and the percentage of two-cell stage embryos was significantly higher in the control group (10.4 ± 1.4%) than in the VEGF-matured group (6.6 ± 0.9%) (Fig. 1). There was no significant difference in the number of eight-cell stage embryos at day 2.

3.2. 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) (Table 2). 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.

3.3. Effects of VEGF on CG distribution in porcine MII oocytes

After 42 h, 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 3, Fig. 2).

4. Discussion

Many reports have suggested that growth factors and cytokines affect mammalian oocytes and embryos during *in vitro* culture [review from 21,22,23]; however, the effects of VEGF supplementation on the *in vitro* maturation of porcine oocytes and subsequent embry-

Table 2
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

Data are given as mean ± SEM from seven replicated experiment. Statistical significant were considered as $P < 0.05$. NS, non significant; S, significant.
[a] percentage of the number of oocytes examined.
[b-d] percentage of the number of oocytes penetrated.

Table 3
CG distribution pattern of porcine MII oocytes after 42 h matured with VEGF (5 ng/mL).

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

* Please see the text for description of CG distribution pattern. Values with different superscripts in same column are significantly different ($P < 0.01$).

onic development after *in vitro* fertilization have not been reported. In the present study, we examined the effects of VEGF treatment on *in vitro* maturation of porcine oocytes, subsequent developmental potential after IVF, and fertilization ability. The cortical granule distribution pattern was evaluated as an indicator of cytoplasmic maturation. We showed that VEGF supplementation during *in vitro* maturation of porcine oocytes significantly increased embryonic developmental potential and the total cell number per blastocyst *in vitro* fertilization compared with the control group. On day 2, although the total cleavage rates did not differ significantly different between the VEGF-matured and control oocytes, the percentage of four-cell stage embryos was significantly higher in VEGF-matured oocytes, and the percentage of two-cell stage embryos was significantly higher in the control group.

The addition of VEGF during *in vitro* maturation of bovine oocytes was reported to accelerate nuclear maturation and promote subsequent embryonic development after *in vitro* fertilization [12]. In our earlier study, we demonstrated that the addition of VEGF during porcine COC IVM significantly increased the intracellular GSH level and embryo developmental rate, and the total cell number per blastocyst was significantly increased after parthenogenetic activation and somatic cell nuclear transfer [15]. Similarly, in another study [24], the microinjection of exogenous reduced or oxidized glutathione into rat embryos significantly increased trophoectoderm development and total cell number as compared with controls, and improved blastocyst damage. The results of the present and previous studies demonstrate that VEGF supplementation during IVM can improve the developmental potential of IVF, parthenogenetic activation, 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.

A relatively high intracellular GSH concentration in oocytes is an indicator of cytoplasmic maturation [25]. GSH is a major sulphydryl-containing protein in mammalian cells and has important biological functions during cellular proliferation, amino acid transport, and DNA and protein synthesis. In addition, GSH helps to protect cells from oxidative damage [26]. After fertilization, intracellular GSH participates in sperm head decondensation, in parallel with oocyte activation. This could explain the higher percentage of four-cell stage embryos in VEGF-matured oocytes and the higher percentage of two-cell stage embryos in the control group. Studies in mice have shown that com-

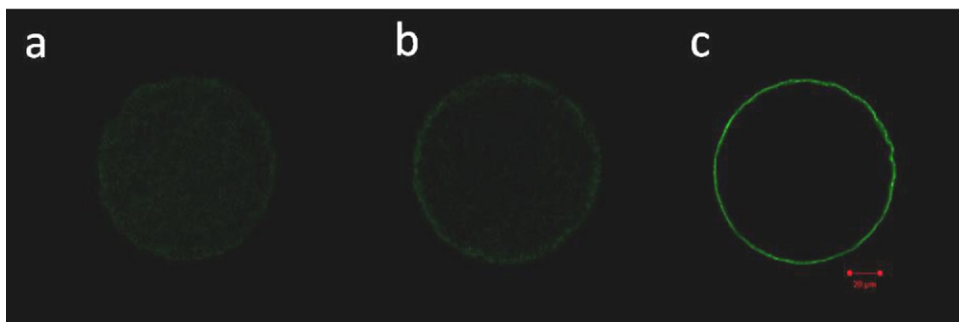

Fig. 2. Confocal images of zona-free pig oocytes stained with FITC-PNA for demonstrating type of CGs distribution in porcine MII oocytes. Type I, complete distribution of CGs throughout the entire ooplasm (a), Type II, partially localized of CGs near to ooplasmic membrane (b), Type III, distribution of CGs in the cortex of cytoplasm aligning with ooplasmic membrane (c). Scale bar = 20 μm.

pared with embryos that develop *in vivo*, fertilized oocytes that develop *in vitro* undergo a two-cell block and have decreased GSH content [27]. Low intracellular GSH concentrations may be responsible for slower developmental competence of porcine oocytes during IVM [28] and high concentration of intracellular GSH may be involved to faster cleavage of porcine zygotes after IVF.

Polyspermic penetration occurs more frequently in pigs than any other species and leads to unresolved problems with respect to IVF [2,29]. Although techniques for achieving *in vitro* maturation and *in vitro* fertilization of pig oocytes have been progressively improved over recent years, polyspermy was still significantly higher in *in vitro* matured oocytes (65%) than those in ovulated oocytes (28%), possibly due to increased aggregation of CGs in ovulated oocytes as compared with *in vitro* matured oocytes [30]. Cytoplasmic maturation is an important factor for preventing polyspermy in mammals. In the present study, polyspermy and sperm penetration per oocyte were significantly reduced in VEGF-matured oocytes as compared with the controls. The penetration of oocytes by more than one spermatozoon is known to be detrimental to further development [6]. However, polyspermic porcine IVM-IVF embryos containing multiple pronuclei can develop *in vitro* to the blastocyst stage at the same percentage as monospermic IVM-IVF embryos. Blastocysts from polypronuclear oocytes have fewer inner cell mass nuclei than blastocysts derived from oocytes with two pronuclei and develop abnormal ploidy, including haploids, triploids, and tetraploids [31]. Interestingly, the transfer of polypronuclear oocytes to a recipient results in pregnancy and the birth of piglets with normal ploidy, indicating that some polypronuclear pig oocytes possess a mechanism to correct ploidy [32].

Monospermy and the MPN formation rate were significantly higher in VEGF-matured oocytes than in the control. In mammalian *in vitro* matured oocytes, intracellular GSH plays an important role in MPN formation after fertilization [32–34]. 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 [35], hamster [36], and pig [25,37]. Intracellular GSH also improves the ability of embryos to alleviate the cytotoxic effects of hydrogen peroxide, which can arrest development *in vitro* at the two-cell stage in mice [38]. Furthermore, high intracellular GSH also appears to be essential for overcoming the *in vitro* developmental arrest in mice and rats embryos [38,39]. Cumulus cells play an important role for intracellular GSH synthesis because the increase in intracellular GSH level is dependent on the presence of cumulus cell mass while denuded oocytes have only a limited capability to synthesis GSH during *in vitro* bovine oocyte maturation [40] and it is initiated by FSH stimulation during *in vitro* porcine oocyte maturation [41]. Optimal cumulus expansion during IVM depends on intracellular GSH contents [42]. Our previous study showed that VEGF treatment during maturation enhances cumulus cell expansion and enhance intracellular GSH synthesis during IVM of porcine oocytes [15].

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 [43,44]. CGs are a specialized group of membrane-bound secretory granules composed of specialized enzymes and glycoproteins that are exquisitely localized near their site of release at non-penetrated oocytes [45,46] and close to their site of action such as the zona pellucida. Thousands of CGs are situated within 2 μm of the plasma membrane of the mature egg, and few are detected in subcortical regions [47]. In mammals, the release of CGs by exocytosis after gamete membrane fusion appears to block polyspermy by changing the properties of the ZP and forming a new layer called "cortical granule envelope" in the perivitelline space [48]. CGs can be visualized using fluorescently labeled lectins. PNA was reported to be a reliable molecular probe for microscopic visualization of CGs in pig oocytes [19], as it specifically binds with sugars on mammalian CGs [49,50]. This method has advantages over the observation of CGs by electron microscopy. Improved techniques with respect to resolution and the ability to optically section the egg have been assessed using laser scanning confocal microscopy [51]. Lectins such as lens culinaris agglutinin bind to CGs in mammalian oocytes [52] and also bind to the ZP of *in vivo* matured porcine oocytes [30]. Thus, the ZP must be removed prior to staining.

In the present study, FITC-PNA was used to stain CGs in zona-free *in vitro* matured oocytes that were matured in the presence or absence of VEGF. 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 [12]. 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.

In conclusion, VEGF supplementation during IVM of porcine oocytes may improve developmental potential by improving the fertilization rate, reducing polyspermy, and improving cortical granule distribution.

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References

[1] Yoshida M, Ishizaki Y, Kawagishi H. Blastocyst formation by pig embryos resulting from in-vitro fertilization of oocytes matured *in vitro*. J Reprod Fertil 1990;88:1–8.
[2] Abeydeera LR. *In vitro* fertilization and embryo development in pigs. Reproduction (suppl) 2001;58:159–73.
[3] Abeydeera LR, Day BN. *In vitro* penetration of pig oocytes in a modified Tris-buffered medium: effect of BSA, caffeine and calcium. Theriogenology 1997;48:537–44.
[4] Wang WH, Hosoe M, Shioya Y. Induction of cortical granule exocytosis of pig oocytes by spermatozoa during meiotic maturation. J Reprod Fertil 1997;109:247–55.
[5] Wortzman GB, Evans JP. Membrane and cortical abnormalities in post-ovulatory aged eggs: analysis of fertilizability and establishment of the membrane block to polyspermy. Mol Hum Reprod 2005;11:1–9.
[6] Hunter RH. Oviduct function in pigs, with particular reference to the pathological condition of polyspermy. Mol Reprod Dev 1991;29:385–91.
[7] Bleil JD, Beall CF, Wassarman PM. Mammalian sperm-egg interaction: fertilization of mouse eggs triggers modification of the major zona pellucida glycoprotein, ZP2. Dev Biol 1981;86: 189–97.
[8] Gospodarowicz D, Abraham JA, Schilling J. Isolation and characterization of a vascularendothelial cell mitogen produced by pituitary-derived folliculo stellate cells. Proc Natl Acad Sci USA 1989;86:7311–5.
[9] Ferrara N, Davis-Smyth T. The biology of vascular endothelial growth factor. Endocr Rev 1997;18:4–25.
[10] Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 1989;246:1306–9.
[11] Dong Q, Cheng Z. Function of VEGF in female reproductive system. Chinease Science Bulletin 2003;48:217–22.
[12] Luo H, Kimura K, Aoki M, Hirako M. Effect of vascular endothelial growth factor on maturation, fertilization and developmental competence of bovine oocytes. J Vet Med Sci 2002; 803–6.
[13] Einspanier R, Schonfelder M, Muller K, Stojkovic M, Kosmann M, Wolf E, Schams D. Expression of the vascular endothelial growth factor and its receptors and effects of VEGF during *in vitro* maturation of bovine cumulus-oocyte complexes (COC). Mol Reprod Dev 2002;62:29–36.
[14] Luo H, Kimura K, Aoki M, Hirako M. Vascular endothelial growth factor (VEGF) promotes the early development of bovine embryo in the presence of cumulus cells. J Vet Med Sci 2002;967–71.
[15] Biswas D, Jeon YB, Kim GH, Jeung EB, Hyun SH. Effect of vascular endothelial growth factor on *in vitro* porcine oocyte maturation and subsequent developmental competence after parthenogenesis. J Anim Vet Adv 2010;9:2924–31.
[16] Hyun SH, Lee G, Kim D, Kim H, Lee S, Nam D, Jeong Y, Kim S, Yeom S, Kang S, Han J, Lee BC, WS Hwang. Production of nuclear transfer-derived piglets using porcine fetal fibroblasts transfected with the enhanced green fluorescent protein. Biol Reprod 2003;69:1060–8.
[17] Yoshioka K, Suzuki C, Tanaka A, Anas IMK, Iwamura S. Birth of piglets derived from porcine zygotes cultured in a chemically defined medium. Biol Reprod 2002;66:112–9.
[18] Koo DB, Kim YJ, Yu I, Kim HN, Lee KK, Han YM. Effects of *in vitro* fertilization conditions on preimplantation development and quality of pig embryos. Anim Reprod Sci 2005;90:101–10.
[19] Yoshida M, Cran DG, Pursel VG. Confocal and fluorescence microscopic study using lectins of the distribution of cortical granules during the maturation and fertilization of pig oocytes. Mol Reprod Dev 1993;36:462–8.
[20] Izadyar F, Hage WJ, Colenbrander B, Bevers MM. The promotory effect of growth hormone on the developmental competence of *in vitro* matured bovine oocytes is due to improved cytoplasmic maturation. Mol Reprod Dev 1998;49:444–53.
[21] Laura DC, George LG. The influence of growth factors on the development of preimplantation mammalian embryos. Arch Med Res 2001;32:619–26.
[22] Richter KS. The importance of growth factors for preimplantation embryo development and in-vitro culture. Curr Opin Obstet Gynaecol 2008;20:292–304.
[23] Stewart CL, Cullinan EB. Preimplantation development of the mammalian embryo and its regulation by growth factors. Dev Genet 1997;21:91–101.
[24] Yuzawa K, Sawada T, Hossain MS, Takagi Y, Hamano K, Tsujii H. Effect of glutathione on the development of rat embryos following microinsemination. Reprod Med Biol 2009;8: 11–7.
[25] Funahashi H, Cantley TC, Stumpf TT, Terlouw SL, Day BN. Use of low-salt culture medium for *in vitro* maturation of porcine oocytes is associated with elevated oocyte glutathione levels and enhanced male pronuclear formation after *in vitro* fertilization. Biol Reprod 1994;51:633–9.

[26] Meister A, Anderson ME. Glutathione. Annu Rev Biochem 1983;52:711–60.
[27] Gardiner CS, Reed DJ. Status of glutathione during oxidant-induced oxidative stress in the preimplantation mouse embryo. Biol Reprod 1994;51:1307–14.
[28] Brad AM, Bormann CL, Swain JE, Durkin RE, Johnson AE, Clifford AL. Glutathione and adenosine triphosphate content of *in vivo* and *in vitro* matured porcine oocytes. Mol Reprod Dev 2003;64:492–8.
[29] Niwa K. Effectiveness of *in vitro* maturation and *in vitro* fertilization techniques in pigs. J Reprod Fertil (Suppl) 1993;48: 49–59.
[30] Wang WH, Abeydeera LR, Prather RS, Day BN. Morphologic comparison of ovulated and *in vitro* matured porcine oocytes, with particular reference to polyspermy after *in vitro* fertilization. Mol Reprod Dev 1998;49:308–16.
[31] Han YM, Abeydeera LR, Kim JH, Moon HB, Cabot RA, Day BN, Prather RS. Growth retardation of inner cell mass cells in polyspermic porcine embryos produced *in vitro*. Biol Reprod 1999;60:1110–3.
[32] Boquest AC, Abeydeera LR, Wang WH, Day BN. Effect of adding reduced glutathione during insemination on the development of porcine embryos *in vitro*. Theriogenology 1999;51:1311–9.
[33] Sutovsky P, Schatten G. Depletion of glutathione during bovine oocyte maturation reversibly blocks the decondensation of the male pronucleus and pronucleus apposition during fertilization. Biol Reprod 1997;56:1503–12.
[34] Zuelke KA, Jeffay SC, Zucker RM, Perreault SD. Glutathione (GSH) concentrations vary with the cell cycle in maturing hamster oocytes, zygotes, and pre-implantation stage embryos. Mol Reprod Dev 2003;64:106–12.
[35] Calvin HI, Grosshans K, Blake EJ. Estimation and manipulation of glutathione levels in prepubertal mouse ovaries and ova: relevance to sperm nucleus transformation in the fertilized egg. Gamete Res 1986;14:265–75.
[36] Perreault SD, Barbee RR, Slott VL. Importance of glutathione in the acquisition and maintenance of sperm nuclear decondensing activity in maturing hamster oocytes. Dev Biol 1988;125:181–6.
[37] Yoshida M. Role of glutathione in the maturation and fertilization of pig oocytes *in vitro*. Mol Reprod Dev 1993;35:76–81.
[38] Nasr-Esfahani MH, Johnson MH. Quantitative analysis of cellular glutathione in early preimplantation mouse embryos developing *in vivo* and *in vitro*. Hum Reprod 1992;7:1281–90.
[39] Slott VL, Hales BF. Effect of glutathione depletion by buthionine sulfoximine on rat embryonic development *in vitro*. Biochem Pharmacol 1987;36:683–8.
[40] de Matos DG, Furnus CC, Moses DF. Glutathione synthesis during *in vitro* maturation of bovine oocytes: role of cumulus cells. Biol Reprod 1997;57:1420–5.
[41] Ozawa M, Nagai T, Somfai T, Nakai M, Maedomari N, Miyazaki H, Kaneko H, Noguchi J, Kikuchi K. Cumulus cell-enclosed oocytes acquire a capacity to synthesize GSH by FSH stimulation during *in vitro* maturation in pigs. J Cell Physiol 2010;222:294–301.
[42] Furnus CC, De Matos DG, Moses DF. Cumulus expansion during *in vitro* maturation of bovine oocytes: relationship with intracellular glutathione level and its role on subsequent embryo development. Mol Reprod Dev 1998;51:76–83.
[43] Ducibella T, Duffy P, Reindollar R, Su B. Changes in the distribution of mouse oocyte cortical granules and ability to undergo the cortical reaction during gonadotropin-stimulated meiotic maturation and aging *in vivo*. Biol Reprod 1990;43: 870–6.
[44] Viana KS, Caldas-Bussiere MC, Matta SG, Faes MR, Paes de Carvalho CS, Quirino CR. Effect of sodium nitroprusside, a nitric oxide donor, on the *in vitro* maturation of bovine oocytes. Anim Reprod Sci 2006;102:217–27.
[45] Cran DG, Esper CR. Cortical granules and the cortical reaction in mammals. J Reprod Fertil Suppl 1990;42:177–88.
[46] Liu XY, Mal SF, Miao DQ, Liu DJ, Bao S, Tan JH. Cortical granules behave differently in mouse oocytes matured under different conditions. Hum Reprod 2005;20:3402–13.
[47] Gulyas BJ. Cortical granules of mammalian eggs. Int Rev Cytol 1980;63:357–92.
[48] Dandekar P, Talbot P. Perivitelline space of mammalian oocytes: extacellular matrix of unfertilized oocytes and formation of cortical granule envelope following fertilization. Mol Reprod Dev 1992;31:135–43.
[49] Ducibella T, Kurasawa S, Rangarajan S, Korf GS, Schultz RM. Precocious loss of cortical granules during mouse oocyte meiotic maturation and correlation with an egg-induced modification of the zona pellucida. Dev Biol 1990;137:46–55.
[50] Long CR, Damiani P, Pinto-Correia C, MacLean RA, Duby RT, Robl JM. Morphology and subsequent development in culture of bovine oocytes matured *in vitro* under various conditions of fertilization. J Reprod Fertil 1994;102:361–9.
[51] Hoodbhoy T, Talbot P. Mammalian cortical granules: contents, fate, and function. Mol Reprod Dev 1994;39:439–48.
[52] Nimura S, Hosoe M. Changes in cortical granule distribution within bovine oocytes during maturation and dertilization *in vitro*. J Reprod Dev 1995;41:103–8.