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Vascular endothelial growth factor A improves quality of matured porcine oocytes and developing parthenotes

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ABSTRACT

Vascular endothelial growth factor is a multipotent angiogenic factor implicated in cell survival and proliferation. The objective was to determine effects of exogenous recombinant human VEGFA (or VEGFA165) in culture media on porcine oocyte maturation and parthenote development. Adding 5 ng/mL VEGFA to the culture medium improved the maturation rate of denuded oocytes ($P < 0.05$), although 5, 50, or 500 ng/mL did not significantly affect nuclear maturation of oocytes. Parthenotes from oocytes cultured either in in vitro maturation or in vitro culture medium supplemented with 5 or 50 ng/mL VEGFA had an improved blastocyst rate and increased total numbers of cells ($P < 0.05$). Moreover, those treated with 5 ng/mL of VEGFA had a higher hatched blastocyst rate (average of 121 cells per blastocyst). All VEGFA-treated oocytes had reduced apoptotic indices ($P < 0.05$), except for those with a higher dose (500 ng/mL) of VEGFA which had more apoptotic cells ($P < 0.05$). Adding 5 ng/mL VEGFA to oocytes during the last 22 h of in vitro maturation improved ($P < 0.05$) blastocyst rates and total numbers of cells, with reduced apoptosis indices similar to that of long-term (44 h) culture. Furthermore, Axitinib (VEGFR inhibitor) reversed the effects of VEGFA on parthenote development ($P < 0.05$). Follicular fluids from medium (2–6 mm) to large (>6 mm) follicles contained 5.3 and 7.0 ng/mL vascular endothelial growth factor protein, respectively, higher ($P < 0.05$) than concentrations in small (<2 mm) follicles (0.4 ng/mL). Also, VEGFA and its receptor (VEGFR-2) were detected (immunohistochemistry) in growing follicles and developing blastocysts. In addition, VEGFA inhibited caspase-3 activation in matured oocytes ($P < 0.05$). In conclusion, this is apparently the first report that VEGFA has proliferative and cytoprotective roles in maturing porcine oocytes and parthenotes. Furthermore, an optimal VEGFA concentration promoted porcine oocyte maturation and subsequent development.

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1. Introduction

Optimized in vitro culture systems for oocytes and embryos are essential to achieve substantial progress in the application of several reproductive biotechnologies. Although in vitro production (IVP) of murine and human embryos has been refined to produce similar cleavage and blastocyst rates compared with their in vivo counterparts [1,2], there is a critical need to improve culture conditions that can maintain high embryo viability in other species, particularly pigs. The latter are considered a suitable source of cells and organs for xenotransplantation, as well as a transgenic animal to produce specific pharmaceutical proteins, given their physiological similarities to humans [3]. Unfortunately, the yield and quality of IVP porcine embryos remain low compared with those of other species and those derived in vivo [4,5]. Specific problems in porcine oocytes and embryos include polyspermy and reduced developmental competence because of insufficient ooplasmic maturation of in vitro maturation (IVM) oocytes and suboptimal embryos with reduced numbers of cells [6–8].

It is well established that amino acids and some macromolecules are required in culture media to support the development of ex vivo embryos [9]. Vascular endothelial growth factors (VEGFs), initially discovered as vascular permeability factors secreted by tumor cells [10], are members of a family that includes VEGFA, VEGFB, VEGFC, VEGFD, placental growth factors, and 2 VEGF-like proteins (encoded by 2 strains of parapoxvirus). It is also well established that VEGFs are crucial regulators for vascular development (vasculogenesis) during embryogenesis, as well as blood vessel formation (angiogenesis) in adults [11]. Members of the VEGF family bind to 3 tyrosine kinase receptors: Flt-1 (VEGFR-1), KDR/Flk-1 (VEGFR-2), and Flt-4 (VEGFR-3) [12]. In that regard, VEGFR-1 binds VEGFA, VEGFB, PLGF1, and PLGF2 to recruit and promote migration of endothelial progenitors and monocytes, whereas VEGFR-2, which is expressed on nearly all endothelial cells, binds VEGFA, VEGFC, VEGFD, and VEGFE, which are involved in regulating endothelial cell proliferation, permeability, survival, and migration. In healthy adults, expression of VEGR3 is mainly limited to lymphatic endothelia, although it may be expressed in tumor-associated blood vessels. By binding to VEGFC and VEGFD, VEGFR-3 is also believed to facilitate outgrowth of lymphatic vessels [13]. Furthermore, VEGFs are important in angiogenic processes during wound healing, ovulation, and pregnancy, as well as in some pathologic conditions, for example, tumorigenesis [13] and glaucoma [14].

In the reproductive system, loss of a single VEGF allele or its receptors (VEGFR-1 and VEGFR-2) is lethal to murine embryos [15]. The VEGFA and/or VEGFR signaling system also regulates germ cell survival and differentiation during spermatogenesis in mice [16]. Furthermore, VEGF and its receptors were detected in the placenta and nonpregnant uterus in pigs [17] and in rats during folliculogenesis [18], whereas placing a VEGF antagonist in the ovarian bursa of rats induced apoptosis, promoting atresia of ovarian follicles [19]. Moreover, VEGF is also involved in the development of ovarian follicles in primates [20–22]. In addition to being a crucial angiogenic factor, VEGF stimulated proliferation and inhibited apoptosis of ovarian follicular cells in cattle [23–25], sheep [26,27], and rats [28]. Considering its multipotency, VEGF has the potential to improve the efficiency of porcine IVP.

In this study, the effects of various doses of VEGFA on maturation of porcine oocytes and subsequent development after parthenogenetic activation were assessed. In addition, VEGFA concentrations in follicular fluid and its signaling pathways in maturing oocytes and developing parthenotes were also investigated.

2. Materials and methods

All chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated.

2.1. Oocyte recovery and IVM

Oocyte recovery and IVM were performed as described [7,29]. Briefly, ovaries obtained from a local abattoir were placed in physiological saline (containing 600 IU/mL penicillin) and transported to the laboratory within 2 h after death. Oocytes were aspirated from follicles 3 to 7 mm in diameter; those with cumulus-oocyte complexes (COCs) with at least 2 layers of cumulus cells and homogeneous ooplasm were selected and placed in NCSU-23 medium for maturation. Twenty to 30 oocytes were randomly allocated to each 100-μL droplet of IVM medium containing various concentrations (0, 5, 50, or 500 ng/mL) of a recombinant human VEGFA or VEGFA165 (R&D System, Minneapolis, MN), covered by mineral oil and cultured at 39°C in an incubator containing 5% CO_2. For the first 22 h, COCs were cultured in NCSU-23 medium supplemented with 10% porcine follicular fluid, cysteine (0.1 mg/mL), equine chorionic gonadotrophin (10 IU/mL), and human chorionic gonadotrophin (10 IU/mL). Thereafter, COCs from all treatment groups were placed in NCSU-23 medium (without exogenous hormones) for another 22 h of IVM.

Determination of meiotic stages was performed 44 h after onset of IVM. Briefly, oocytes were completely denuded via gently pipetting with a fine bore glass pipette and a solution of 0.1% (wt/vol) hyaluronidase in Dulbecco phosphate-buffered saline (DPBS). The oocytes were then fixed in a 3.7% formaldehyde-Triton X-100 (Sigma) solution for 30 min, washed in DPBS for an additional 3 min, mounted on a slide, and stained with 1.9 μM Hoechst 33,342 in anti-bleaching DAKO fluorescent mounting medium (DAKO Corporation, Carpinteria, CA). Stained oocytes were examined at 400× magnification using an epifluorescent microscope equipped with a wide range pass UV (Olympus AX70) to determine their stage of meiosis classified as germinal vesicle, germinal vesicle breakdown, metaphase I, and metaphase II (MII).

2.2. Oocyte activation and embryo culture

After 44 h of IVM, matured oocytes were stripped from cumulus cells by gentle pipetting in DPBS containing 0.1% hyaluronidase. Oocytes with an extruded polar body were selected and washed twice in activation medium (0.28 M mannitol, 0.01% polyvinyl alcohol, 0.05 mM HEPES, 0.1 mM $CaCl_2 \cdot 2H_2O$, and 0.1 mM $MgCl_2$) before parthenogenetic activation. For the latter, an electrical pulse (2.04 kV/cm, 30 μs) generated by a BTX Electro-Cell Manipulator 2001 (BTX, San Diego, CA) was applied. Thereafter, oocytes were washed twice with porcine zygote medium (PZM-3) containing 2.5 mM 6-dimethylaminopurine and transferred to the same pre-incubated medium for 4 h. Finally, activated oocytes were washed 6 times with PZM-3 medium before being cultured continuously (7 d) in PZM-3 medium, following the same treatment described previously, to evaluate developmental competence, as described [7,29,30].

2.3. Effects of VEGFR inhibition on development of parthenogenetic embryos

Axitinib (AG-013736, Biovision Incorporated, Milpitas Boulevard, Milpitas, CA) is a small molecule tyrosine kinase inhibitor. Its primary mechanism of action is believed to be inhibition of VEGFR-1, VEGFR-2, VEGFR-3, c-KIT, and PDGFR. To determine whether the observed effects could be attributed to VEGFA, groups of COCs or activated oocytes were incubated for 1 h with 10 ng/mL of Axitinib before being allocated to NCSU-23 or PZM-3 (in the presence of VEGFA), with or without Axitinib or without VEGFA (control) at 39°C in an incubator containing 5% CO_2, as described [7,31], with slight modifications.

2.4. Apoptosis assays and total cell counts

Apoptosis was detected with a terminal deoxynucleotidyl transferase-mediated d-UTP nick end-labeling (TUNEL) assay, as described [7,29]. Briefly, 7-d-old embryos were washed 3 times in DPBS/PVP (DPBS supplemented with 0.1% polyvinylpyrrolidone) and fixed in 4% (vol/vol) paraformaldehyde solution for 1 h at room temperature, and then the embryos were placed in 0.1% Triton X-100 and 0.1% citrate solution for 1 h at room temperature (to increase permeability). Thereafter, embryos were incubated in TUNEL reaction medium (In Situ Cell Death Detection Kit, Fluorescein; Roche, Mannheim, Germany) for 1 h at 38.5°C in the dark. In the process, broken DNA ends within embryonic cells were labeled with TdT and fluorescein-dUTP. After the reaction was stopped, embryos were washed in DPBS/BSA (DPBS supplemented with 0.1% bovine serum albumin) and mounted on glass slides with DAKO Fluorescent Mounting Medium (S3023, Dako North America, Carpinteria, CA) containing Hoechst 33,342. Whole-mount embryos were examined under an epi-fluorescence microscope (Nikon) by TUNEL assay and Hoechst staining. Numbers of apoptotic nuclei (TUNEL assay) and total numbers of nuclei were determined from optical images. The apoptotic index was calculated as follows:

$$\textit{Apoptotic index}\,\% = (\textit{number of TUNEL} - \textit{positive nuclei} / \textit{total number of nuclei}) \times 100\%$$

2.5. VEGF protein quantification

Follicular fluid concentrations of VEGFA proteins were measured with a highly specific sandwich enzyme-linked immunosorbent assay that recognized VEGFA165 and VEGFA121 (Quantikine; R&D Systems, Minneapolis, MN), as reported [32].

2.6. Immunocytochemistry

To localize VEGFR-2, samples were collected (from newly harvested COCs to 7-d embryos) and processed as follows: samples were fixed in 4% paraformaldehyde for 30 min at room temperature, washed 3 times in PBS containing 2% BSA (PBS-BSA), and then permeabilized in PBS with 0.25% Triton X-100 (Sigma-Aldrich) at room temperature for 1 h. After 3 washes in PBS-BSA, they were transferred into blocking solution (PBS supplemented with 10% normal goat serum, 2% BSA, 2% non-fat dried milk, and 0.15 M glycine) for 1 h at 37°C, and then incubated with the primary antibody of anti-VEGFR-2 clone 55B11 rabbit monoclonal antibody (#2479; Cell Signaling Technology, Danvers, MA) in PBS-BSA at 4°C overnight. Thereafter, samples were rinsed 3 times and incubated in PBS-BSA in the presence of anti-rabbit fluorescein isothiocyanate-conjugated anti-rabbit IgG (Sigma-Aldrich, Co; 1:50 dilution) at 37°C for 1 h followed by 4 rinses, before mounting in a medium containing Hoechst 33,342 (S3023, Dako North America) for nuclear staining. The negative control was performed concurrently with PBS-BSA in lieu of the primary antibody (all other steps were identical). If immunocytochemical labeling is successful, blastomeres express red fluorescence on their plasma membranes and nuclei are blue [27,33].

2.7. Immunohistochemical staining of ovarian tissue sections

Ovarian tissue sections were stained immunohistochemically as described [18,23,34]. Briefly, fresh ovaries were fixed in 4% paraformaldehyde in DPBS, washed twice in DPBS and dehydrated through a gradient series of ethyl alcohol before being embedded in Paraplast embedding media (Sigma-Aldrich). Histological sections (2 μm) were produced with a microtone (Leica RM 2145, Nussloch, Germany). Sections were processed for antigen retrieval by boiling in citrate buffer (pH 6.0) for 5 min and then treated with blocking buffer prepared in 1 × TBS (20 mM TRIS-HCl, 500 mM NaCl, 0.05% Tween 20, 1% BSA, 0.3% Triton X-100, and 1% normal horse serum). After 4 h incubation in blocking buffer, sections were incubated for another 4 h in the presence of a polyclonal primary antibody (rabbit anti-VEGF, Cat. No. orb77281; Biorbyt, Cambs, UK) or anti-VEGFR-2 (Cat. No. 2472; Cell Signaling Technology Inc,

Beverly, MA) at working dilutions (VEGF, 1:300; VEGFR-2, 1:200). After 3 washes in TBS, slides were incubated in the presence of horseradish peroxidase-conjugated secondary antibodies (Jackson Immuno Research, West Grove, PA) at 4°C for 3 h. Slides were then washed 3 times with TBS, followed by color development with diaminobenzidine kits (Laboratory Vision, Freemont, CA) and hematoxylin for counterstaining. The negative controls, without adding primary antibody for VEGFA and VEGFR-2, were obtained from human tumor mass samples. Ovarian tissues were observed with light microscopy (BX-51, Olympus, Tokyo, Japan) and micrographs were recorded with a digital camera (DP20, Olympus).

2.8. Western blotting

Western blot analysis was performed as described [7,34]. The COCs were cultured with or without VEGFA supplementation (0, 5, 50, or 500 ng/mL) during IVM. After incubation for 44 h, oocytes were denuded of cumulus cells and washed 3 times in DPBS containing 0.1% polyvinyl alcohol (PVA, P-8136). Fifty matured oocytes per treatment per replicate were collected into sample buffer. Samples were collected and stored at −80°C in sample buffer (100 mM Tris-HCl 200 mM β-mercaptoethanol, 0.4% sodium dodecyl sulfate, 0.002% bromophenol blue, and 20% glycerol) pending further studies. For electrophoresis, samples were boiled for 5 min, cooled in ice, and then loaded on 10% SDS-PAGE gels. Proteins were transferred from the gel to nitrocellulose membranes (Cat. No. HAHY0010, Millipore, Billerica, Ireland) and blocked for 1 h in TBST buffer (20 mM Tris-HCl, 500 mM NaCl, and 0.1% Tween 20) containing 5% chicken serum. Membranes were then incubated overnight at 4°C with primary antibodies: caspase-3 (1:500; Cat. No. 9662; Cell Signaling Technology Inc) and β-actin (1:1000, Cat. No. 4967; Cell Signaling Technology Inc). After incubation, nitrocellulose membranes were washed 5 times and then incubated (for 1 h at room temperature) with secondary antibody (1:10,000; horseradish peroxidase-labeled anti-rabbit or anti-mouse immunoglobulin as appropriate; Cell Signaling Technology Inc). After 3 washes, proteins were detected with the Super Signal West Pico Chemiluminescent Subs Kit (Pierce Biotechnology, Inc, Rockford, IL) and visualized on an X-ray film. Band intensities were measured using ImageJ software (Version 1.44p; National Institute of Health, Bethesda, MD), and were normalized (for statistical analyses) to β-actin level.

2.9. Statistical analyses

All percentile data were normalized by arcsine transformation before statistical analysis. Differences among treatments for cleavage rates, total numbers of cells, percentages of blastocysts, and apoptotic indices were tested by the general linear model procedure (SAS Institute, Cary, NC). Differences between treatment means were located with a Tukey test and were considered significant at $P < 0.05$. All data were expressed as mean ± standard error of the mean.

3. Results

3.1. Oocyte maturation

This experiment was designed to evaluate oocyte maturation in terms of polar body extrusion, arresting at metaphase II. None of the treatment significantly affected nuclear maturation stages of oocytes including germinal vesicle, germinal vesicle breakdown, metaphase I, and MII. However, 5 ng/mL of VEGFA improved the maturation rate (MII) of denuded oocytes (Fig. 1, $P < 0.05$) compared with other treatment groups.

3.2. Effects of VEGFA supplementation in maturation medium on parthenote development

In the experiment, the effect of VEGFA supplementation in maturation medium on parthenote development was tested. Results showed that 5 and 50 ng/mL groups had greater ($P < 0.05$) blastocyst rates and total numbers of cells (compared with the control or 500 ng/mL VEGFA; Table 1).

Furthermore, all VEGFA-treated groups had a reduced ($P < 0.05$) apoptosis index compared with the control group, whereas embryos supplemented with 5 ng/mL of VEGFA for the first 22 h of IVM had increases ($P < 0.05$) in blastocyst rate and total number of cells per blastocyst and a reduction ($P < 0.05$) in apoptosis (Table 2).

3.3. Effects of VEGFA in culture medium on parthenote development

This experiment was designed to evaluate the effect of VEGFA supplementation on porcine parthenote development. Blastocyst rates were higher ($P < 0.05$) in groups cultured with 5 or 50 ng/mL VEGFA compared with either the control or 500 ng/mL groups (Table 3). Furthermore, all VEGFA-treated groups had greater ($P < 0.05$) total numbers of cells per blastocyst compared with the control group. The addition of 50 ng/mL of VEGFA resulted in a lower ($P <$

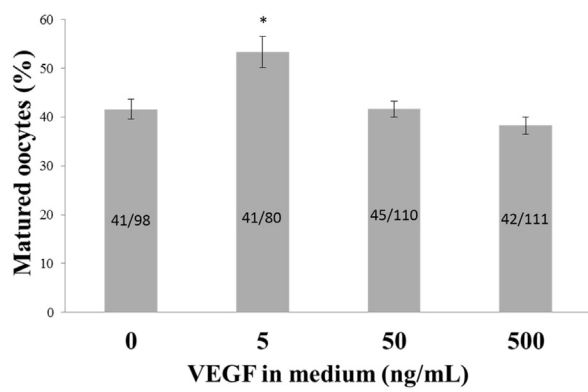

Fig. 1. Effects of adding VEGFA to the maturation medium on polar body extrusion of denuded porcine oocytes. The asterisk on the column indicates that the maturation rate in the 5 ng/mL group was higher than that in other groups ($P < 0.05$). Five replicates. Numbers on the bars are the total number of oocytes with extruded polar body/total number of matured oocytes. MII, metaphase II; MI, metaphase I; GVBD, germinal vesicle breakdown; GV, germinal vesicle.

Table 1
Development of porcine parthenotes in IVM medium supplemented with various concentrations of VEGFA. Data are mean ± SEM (5 replicates). All developmental rates are in percentages.

VEGFA (ng/mL)	Total number of oocytes	Cleavage rate (n)	Blastocyst rate (n)	TCN/blastocyst (n)	Apoptosis index
0	141	92.78 ± 1.48 (131)	40.33 ± 1.08a (57)	44.87 ± 1.41a (45)	7.39 ± 1.62a
5	148	91.12 ± 1.43 (135)	53.61 ± 3.13b (78)	61.52 ± 2.92b (47)	2.91 ± 0.74b
50	130	94.54 ± 0.76 (123)	49.35 ± 2.28ab (64)	66.77 ± 6.96b (42)	2.67 ± 0.44b
500	132	95.26 ± 1.45 (125)	39.75 ± 3.31a (51)	52.90 ± 2.24a (43)	3.31 ± 1.00b

Abbreviations: IVM, in vitro maturation; SEM, standard error of the mean; TCN, total cell number.
Within a column, means without a common letter (a,b) differed ($P < 0.05$).

Table 2
Development of porcine parthenotes cultured in medium supplemented with an optimized concentration of VEGFA during the first 22 h of IVM. Data are mean ± SEM (5 replicates). All developmental rates are in percentages.

VEGFA (ng/mL)		Total number of oocytes	Cleavage rates (n)	Blastocyst rate (n)	TCN/blastocyst (n)	Apoptosis index
IVM(+)	IVM(−)					
0	0	135	93.88 ± 1.05 (127)	43.07 ± 0.84 (63)a	46.54 ± 0.99a (41)	6.14 ± 0.49a
5	0	136	94.06 ± 1.44 (128)	52.75 ± 2.29 (69)b	59.18 ± 1.20b (46)	4.13 ± 0.51b
0	5	137	92.71 ± 1.44 (127)	46.16 ± 03.13 (63)ab	47.69 ± 1.25a (50)	5.12 ± 0.86ab
5	5	123	93.23 ± 2.09 (117)	52.95 ± 2.35 (64)b	54.47 ± 0.80c (46)	3.05 ± 0.34b

Abbreviations: IVM, in vitro maturation; SEM, standard error of the mean; TCN, total cell number.
Within a column, means without a common letter (a,b,c) differed ($P < 0.05$).

Table 3
Development of pig parthenotes in the embryo culture medium supplemented with various concentrations of VEGFA. Data are mean ± SEM percentage (6 replicates). All developmental rates are in percentages.

VEGFA (ng/mL)	Total number of oocytes	Cleavage rate (n)	Blastocyst rate (n)	TCN/blastocyst (n)	Apoptosis index
0	143	88.11 ± 2.75 (126)	34.24 ± 6.24a (49)	48.75 ± 1.50a (47)	4.72 ± 0.36a
5	145	87.59 ± 3.15 (127)	56.45 ± 3.45b (82)	66.28 ± 3.24b (69)	3.37 ± 0.41ab
50	138	84.78 ± 3.81 (117)	55.07 ± 4.75b (76)	62.90 ± 3.46b (61)	2.25 ± 0.47b
500	137	88.32 ± 3.90 (121)	37.23 ± 3.14a (51)	58.30 ± 3.95b (45)	3.00 ± 0.71ab

Abbreviations: SEM, standard error of the mean; TCN, total cell number.
Within a column, means without a common letter (a,b) differed ($P < 0.05$).

Table 4
Development of pig parthenotes when IVM and IVC media are both supplemented with various concentrations of VEGFA. Data are mean ± SEM percentage (6 replicates). All developmental rates are in percentages.

VEGFA (ng/mL)	Total number of oocytes	Cleavage rate (n)	Blastocyst rate (n)	TCN/blastocyst (n)	Apoptosis index
0	143	94.40 ± 1.72 (135)	37.06 ± 2.33a (53)	42.47 ± 03.19a (40)	5.88 ± 0.07a
5	144	93.05 ± 2.35 (134)	42.36 ± 2.66ab (61)	52.37 ± 0.72b (47)	3.08 ± 0.13b
50	144	94.44 ± 2.27 (136)	50.00 ± 2.04b (72)	64.39 ± 01.99c (47)	3.09 ± 0.22b
500	133	93.23 ± 2.00 (124)	35.34 ± 2.29a (47)	52.21 ± 03.79b (39)	6.54 ± 0.20a

Abbreviations: IVC, in vitro culture; IVM, in vitro maturation; SEM, standard error of the mean; TCN, total cell number.
Within a column, means without a common letter (a,b,c) differed ($P < 0.05$).

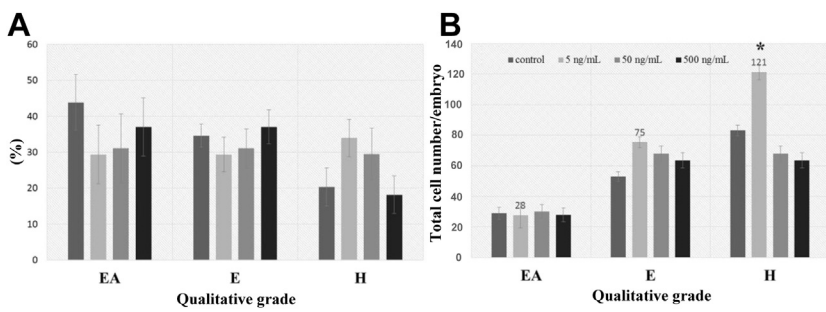

Fig. 2. Distribution of porcine embryos in various qualitative grades (A) and total numbers of cells per parthenogenetically derived blastocyst (B) cultured in medium supplemented with various doses of VEGFA (0, 5, 50, or 500 ng/mL) for 7 d. *Within each category, a column with an asterisk differed ($P < 0.05$). Total numbers of blastocysts used were 62, 81, 68, and 54 for 0, 5, 50, and 500 ng/mL, respectively. Five replicates. Differential qualitative grades: H, hatched blastocyst; E, expanded blastocyst; EA, early blastocyst.

Table 5
Development of pig parthenotes cultured in the medium containing an optimized VEGFA[a] concentration with or without inhibitor Axitinib[b]. Data are mean ± SEM percentage (7 replicates). All developmental rates are in percentages.

VEGFA (ng/mL)	Total number of oocytes	Cleavage rate (n)	Blastocyst rate (n)	TCN/blastocyst (n)	Apoptosis index
Ctrl	156	80.96 ± 3.3 (126)	35.82 ± 1.9a (55)	55.94 ± 6.3a (43)	5.57 ± 0.8a
VEGF	156	88.86 ± 3.0 (138)	48.61 ± 3.1b (75)	79.03 ± 5.7b (53)	2.25 ± 0.5b
VEGF + Axitinib	156	81.36 ± 3.4 (127)	35.96 ± 2.5a (56)	60.2 ± 11.2a (42)	4.61 ± 1.0ab
Axitinib	156	80.75 ± 2.6 (126)	33.32 ± 1.9a (52)	54.95 ± 6.7a (37)	4.59 ± 0.84ab

Abbreviations: SEM, standard error of the mean; TCN, total cell number; VEGF, vascular endothelial growth factor.
Within a column, means without a common letter (a,b) differed ($P < 0.05$).
[a] VEGF: 5 ng/mL.
[b] Axitinib: 10 ng/mL.

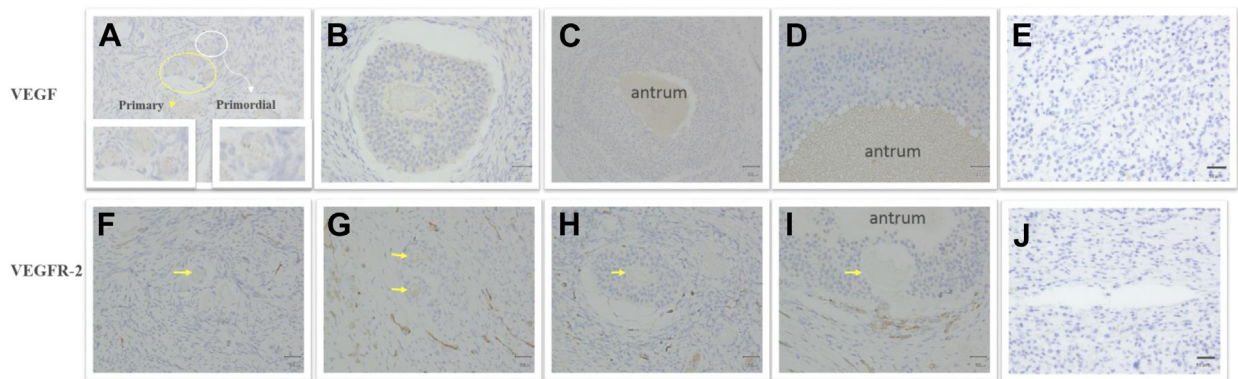

Fig. 3. Expressions of VEGF (A–D) and VEGFR-2 (E–J) in porcine ovarian follicles at various developmental stages (detected by immunohistohemical staining). Primordial and primary follicles (A) had immune reactivity in the oocyte, as did secondary follicles (B). In tertiary follicles (C), both the oocyte and follicular fluid were positive in expressing VEGF and its receptor. Larger follicles (D) had more intense immunoreactivity. The VEGFR-2 was immunopositive on the oocyte membrane in clusters of primordial follicles (F), primary follicles (G), secondary follicles (H), and tertiary follicles with the cumulus-oocyte complex (I). E and J are representative negative controls from tumor mass samples where the primary antibodies were not added. Yellow arrow indicates the oocyte in the follicle. Scale bar: 50 μm. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

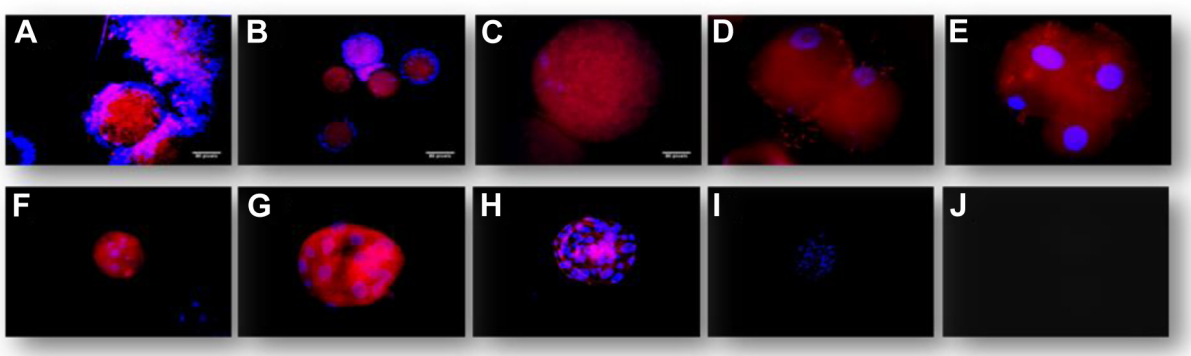

Fig. 4. Representative images of the VEGFR-2 detected by immunocytochemistry at various development stages. After fixation in 4% paraformaldehyde, VEGFR-2 was detected using an anti-VEGFR-2. Positive immunoreaction (red) was present in newly collected cumulus-oocyte complexes (A), denuded oocytes (B), metaphase II oocytes (C), and 2-cell (D), 4-cell (E), 8-cell (F), morula (G), and blastocyst stage embryos (H). (I, J) Embryos for negative control (with secondary antibody, without primary antibody). Nuclei stained with Hoechst were blue. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

0.05) apoptotic index compared with the control group (Table 4).

3.4. Effects of VEGFA addition to IVM and IVC media on parthenogenetic embryo development

In this experiment, the effects of VEGFA supplementation in both the maturation and culture media on the developmental competence of porcine parthenotes were examined. Parthenotes supplemented with 50 ng/mL VEGFA had a greater ($P < 0.05$) blastocyst rate compared with the control group. Embryos grown in all VEGFA-supplemented media also had a greater ($P < 0.05$) total number of cells per blastocyst, whereas only those supplemented with 5 or 50 ng/mL VEGFA had lower apoptotic indices ($P < 0.05$). To differentiate the quality of 7-

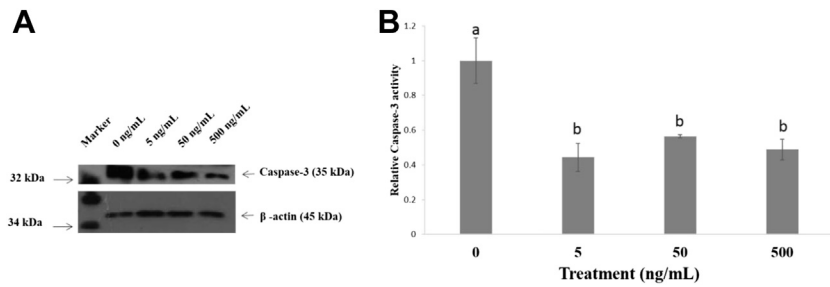

Fig. 5. Analysis of the VEGFA165 signaling-related molecules in matured porcine oocytes. Representative immunoblots with (A) caspase-3 and β-actin antibodies. (B) Relative expression of caspase-3 to β-actin (an internal control) from oocytes matured in the medium containing various doses of VEGFA165 protein (0, 5, 50, or 500 ng/mL).

d blastocysts, blastocyst embryos were arbitrarily classified (on the basis of gross morphology) into 3 groups: early (EA), expended (E), and hatched (H) embryos. The group exposed to 5 ng/mL VEGFA had the most hatched blastocysts (Fig. 2A) and the greatest total number of cells (Fig. 2B).

3.5. Effects of VEGFR inhibition during the culture period of embryos

This experiment was designed to confirm the effect of VEGFA on porcine embryo development using parthenotes by blocking VEGFR with its specific inhibitor. When Axitinib, an inhibitor of VEGFR-1, VEGFR-2, and VEGFR-3, was present in the culture medium, it negated the effect of VEGFA to that of control group, vis-à-vis the blastocyst rate and total numbers of cells (Table 5). Furthermore, it was noteworthy that 10 ng/mL Axitinib in culture medium did not have any apparent toxicity (development of parthenotes approximated that of the control group).

3.6. VEGFA and its receptor (VEGFR-2)

This experiment was to provide another evident parameter to support the presence of VEGFA and its receptor in the ovary, cumulus-oocyte complexes and developing porcine parthenotes by enzyme-linked immunosorbent assay analysis. Concentrations of VEGF in follicular fluids derived from medium (2–6 mm) and large (>6 mm) follicles were 5395.3 ± 639.4 and 6927 ± 498.0 pg/mL, respectively, both of which were higher ($P < 0.05$) than the concentrations in small (<2 mm) follicles (452.4 ± 229.7 pg/mL). Based on immunohistochemistry, oocytes in primordial, primary, secondary, and tertiary follicles all had positive VEGF signals (Fig. 3). Furthermore, VEGF protein was detected in follicular fluids and immunoreactivity increased with follicle diameter. Concomitantly, VEGFR-2 was detected in oocytes from primordial to tertiary follicles. Positive VEGFR-2 was also detected in newly harvested COCs, matured oocytes, and developing embryos (up to the blastocyst stage; Fig. 4).

3.6.1. Activation of signaling molecules

This experiment was to assess the activity of downstream molecules inherent to VEGFA signaling. Caspase-3 activation was reduced ($P < 0.05$) in all VEGFA-treated groups (5, 50, and 500 ng/mL) compared with the control group (0 ng/mL) at levels of 0.44, 0.56, and 0.49 versus 1, respectively (Fig. 5).

4. Discussion

It is well established that VEGF has a variety of physiological functions including some in the reproductive system and folliculogenesis. In the present study, exogenous VEGF was added to culture media to assess its effects on porcine oocyte maturation and parthenote development. Expressions of VEGFA and VEGFR-2 were confirmed in porcine ovarian follicles in various stages of development. Furthermore, the PI3K/AKT and ERK/MEK signaling pathways mediating VEGF proliferative and cytoprotective effects were also detected.

Adding VEGF to IVM medium did not ameliorate polar body extrusion rate in COCs, similar to bovine COCs [23], but in contrast to reports of cultured porcine [35] and ovine [27] embryos using TCM199-based medium. However, that VEGF increased extrusion of polar bodies in denuded oocytes, we inferred that there may be VEGF receptors on oolemma membranes. The addition of VEGFA (5 or 50 ng/mL) either in maturation or culture media, improved blastocyst rates and embryo quality through amelioration of total numbers of cells and reduction of apoptotic indices. The effective dose seemed to be species-dependent, as caprine follicles grew well with 10 to 200 ng/mL VEGF [36], whereas 300 ng/mL was appropriate for bovine embryos [23].

When cultured continuously in the presence of 5 ng/mL VEGFA, hatching rate and total cell numbers (121 cells/blastocyst) were substantially increased in the present study. We inferred that these embryos had approximately 7 cleavages ($2^7 = 128$), with reduced occurrence of cell death. It was noteworthy that even addition of VEGFA (5 ng/mL) only for the first 22 h of IVM enhanced blastocyst rates, total numbers of cells, and reduced apoptosis of embryonic cells. Not only were the mitogenic properties of VEGFA demonstrated in this study, but also its cytoprotective roles were also apparent, based on the improvement of total numbers of cells and reduction of apoptotic indices. Beneficial effects of VEGFs on preimplantation blastocysts have been reported in cattle [23,25], water buffalo [37],

goats [36], pigs [35], sheep [27], and rats [28,38]. Furthermore, it is well established that VEGFs induce proliferation and survival of endothelial cells [39–41].

Axitinib (10 ng/mL) per se did not have any apparent adverse effects on embryo development in the present study. However, it negated the benefits of VEGF when both were added to the culture medium. Axitinib is a small molecule tyrosine kinase inhibitor for a group of growth factors or molecules including VEGFR-1, VEGFR-2, VEGFR-3, platelet derived growth factor receptor (PDGFR), and c-KIT (CD117). It acts by competing for adenosine triphosphate-binding to the intracellular kinase domain [41]. Therefore, we concluded that VEGF was the ligand responsible for the improvements that were detected.

In the present study, oocytes expressed VEGF and VEGFR-2 from primordial to antral follicle stages; furthermore, their concentrations in the follicular fluid also increased as the antrum became larger. Similar increases in follicular VEGF concentrations with increasing follicle size have been reported in rats [24] and pigs [32] given exogenous gonadotropins. Using immunocytochemistry, VEGFR-2 were detected in newly harvested COCs, matured oocytes, and developing parthenogenetic embryos, similar to previous reports [27,42]. Araujo [43] demonstrated that growth factors have important roles in the female reproductive system, particularly regulating differential ovarian functions, that is, ovarian cell proliferation, apoptosis, folliculogenesis, luteogenesis, oogenesis, secretion of hormones, responses to hormonal regulators, fertility, and, in some cases, development of ovarian disorders. Among the panoply of growth factors, VEGFA is crucial for physiological functions and spatiotemporal expression in the reproductive system [16,34]. In that regard, VEGF and its receptors are expressed in a wide spectrum of cells or tissues, including ovarian follicular cells [18,24,37,43], oviducts, endometria during implantation [44], and menstruation/post-menstruation repair [45], as well as endometrial cells during pregnancy [46], placenta and nonpregnant uteri [17], and the conceptus or fetus during preimplantation and peri-implantation periods [15,46]. Furthermore, VEGF also has a crucial role in survival of rat ovarian cells [28]; its expression in oocyte zona pellucida (secondary follicles) has been used to predict follicle viability [37]. Recruitment and selection of ovarian follicles during the early follicular phase in non-human primates was also regulated by VEGFA/VEGFR-2, with disturbances delaying follicular development [20,47]. Injection of VEGF into porcine [21] or rat [22] ovaries increased the number of pre-ovulatory follicles by preventing them from undergoing atresia. Furthermore, local inhibition of VEGFA activity increased ovarian apoptosis through an imbalance among BCL2 family members, leading to more follicles undergoing atresia [19]. Gerber et al [48] demonstrated that VEGFs not only exerted their effects by a paracrine mechanism but also by an autocrine loop. In addition, ovine oocytes can increase cytoplasmic VEGF content and subsequently expression of VEGFRs when cultured in medium supplemented with VEGF [27]. The presence of VEGFA and its receptor in all developmental stages during folliculogenesis strengthened its role in regulating follicle destiny, whereas detection of VEGFR-2 implicated it in mediating VEGF function.

Biochemical pathways underlying the beneficial effects of VEGFA on porcine oocytes during IVM have not been fully elucidated. In the present study, VEGFA induced reductions in caspase-3 activity in VEGFA-treated oocytes. It has been reported that VEGFA exerted proliferative and cytoprotective effects through the PI3K/AKT and ERK/MEK signaling pathways in endothelial cells [39–41], tumor cells [49], retinal progenitor cells [50], rat follicular granulosa cells [25,28], porcine theca cells [51], and ovine in vitro oocytes [27]. Inhibiting caspase-3 activity was associated with rescuing granulosa cells from undergoing apoptosis and prevented atresia of bovine ovarian follicles [24]. Survivin, a baculoviral inhibitor of apoptosis repeat-containing 5 (BIRC5), was co-expressed with VEGF [52,53], suggesting that the effects of VEGFA effect may be mediated by an enhanced expression of other intermediate molecules. However, additional studies are needed.

In conclusion, VEGFA supplementation in maturation medium enhanced oocyte maturation even in denuded oocytes. For embryonic development, it improved quality and promoted development of porcine parthenotes, likely by stimulation of blastomere proliferation and inhibition of apoptosis (when added at an appropriate concentration). Further study is required to elucidate whether the function of VEGFA/VEGFRs during follicle development is mediated by the PI3K/AKT and ERK/MEK signaling.

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