Presence of vascular endothelial growth factor during the first half of IVM improves the meiotic and developmental competence of porcine oocytes from small follicles

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The aim of the present study was to investigate the effect of vascular endothelial growth factor (VEGF) on the meiotic and developmental competence of porcine oocytes from small follicles (SF; 0.5–3 mm diameter). When cumulus–oocyte complexes (COCs) from medium-sized follicles (MF; 3–6 mm diameter) and SF were cultured for IVM, the maturation rates were significantly higher for oocytes from MF than SF. Concentrations of VEGF in the medium were significantly higher for COCs cultured from MF than SF. When COCs from SF were exposed to 200 ng mL^{-1} VEGF during the first 20 h of IVM, the maturation rate improved significantly and was similar to that of oocytes derived from MF. The fertilisability of oocytes was also significantly higher than that of VEGF-free SF controls. Following parthenogenetic activation, the blastocyst formation rate improved significantly when SF COC culture was supplemented with 200 ng mL^{-1} VEGF, with the rate similar to that of oocytes from MF. The results of the present study indicate that VEGF markedly improves the meiotic and developmental competence of oocytes derived from SF, especially at a concentration of 200 ng mL^{-1} during the first 20 h of IVM.

Additional keyword: pig.

Ovaries contain a large number of small follicles, but the meiotic and developmental competence of oocytes from these follicles is quite low. In the present study we demonstrated that the addition of 200 ng mL^{-1} vascular endothelial growth factor to the IVM medium increased the maturation rate of porcine oocytes from small follicles and that blastocyst formation following parthenogenetic activation also increased. These findings may contribute to efficient animal production and human assisted reproductive technology.

Introduction

Using IVM oocytes to produce embryos is a common practice that is essential for applications such as increasing opportunities for the successful birth of babies in assisted reproductive medicine, reducing the generation interval in important species and producing transgenic animals for cell therapies, protein production or other medical applications (Galli *et al*. 2003; Cooper *et al*. 2013). However, only cumulus–oocyte complexes (COCs) derived from medium-sized follicles (MF; 3–6 mm diameter) or larger follicles

have been used for IVM and IVF; oocytes derived from small follicles (SF; <3 mm diameter), which account for most follicles in ovaries, have been found to have much lower competence to mature to MII *in vitro* (Yoon *et al*. 2000; Romaguera *et al*. 2010*a*; Kohata *et al*. 2013) because of a lack of factors that regulate meiotic and cytoplasmic maturation (Romaguera *et al*. 2010*b*). Therefore, if we can improve the meiotic and developmental competence of oocytes derived from SF, we may be able to use more follicular oocytes per ovary to produce embryos *in vitro*, consequently contributing to the development of animal production and human assisted reproductive technologies (ARTs). Because porcine ovaries contain a relatively larger number of follicular resources than other species, including humans (Gosden and Telfer 1987), the pig is one of the suitable species in which the meiotic and developmental competence of oocytes derived from SF can be studied.

Angiogenesis plays an important role in the mechanism of selection and development of ovarian follicles (Bruno *et al*. 2009). In the ovary, vascular endothelial growth factor (VEGF) has been identified as promoting (Leung *et al*. 1989) and being involved in the regulation of normal or abnormal angiogenesis (Ferrara *et al*. 2003). This protein was first identified as vascular permeability-inducing factor (VPF) secreted by tumour cells (Senger *et al*. 1983). VEGF is a 34- to 42-kDa protein, and there are seven members of the VEGF family. VEGF interacts with its receptors present in granulosa and theca cells to act as a mitogenic factor in developing goat (Bruno *et al*. 2009) and preantral human follicles (Abir *et al*. 2010).

The expression and levels of VEGF and its receptors (VEGFR2) are upregulated as the follicle develops (Greenaway *et al*. 2004). The presence of VEGF allows development of the vascular network and induces cell proliferation, whereas a lack of VEGF results in regression of vessels in non-productive ovarian follicles, likely atresia (Hanahan 1997). Inactivation of the *Vegf* gene causes abnormal development of angiogenesis and reduces the viability of murine embryos (Ferrara *et al*. 1996). In addition, blocking the Flk/KDR pathway demonstrated that VEGF was involved in the delay of folliculogenesis in rhesus monkey (Zimmermann *et al*. 2002). In addition, concentrations of VEGF in bovine follicular fluid have been found to be fivefold higher in dominant follicles just before ovulation compared with early antral follicles (Einspanier *et al*. 2002). Together, these findings suggest that VEGF is involved in the acquisition of meiotic and developmental competence by oocytes.

It has been reported that supplementing the culture medium with VEGF has stimulatory effects on the quality of mature porcine oocytes derived from MF and their developmental competence following parthenogenetic activation (Kere *et al*. 2014). In the bovine, exposure of COCs derived from MF to 5 ng mL^{-1} VEGF during the first 20 h of IVM resulted in a higher percentage of matured oocytes, normal fertilisation and increased blastocyst yield, suggesting that VEGF may induce not only nuclear

maturation, but also cytoplasmic maturation (Luo *et al*. 2002). Similar results were reported recently with concentrations of 300–500 ng mL^{-1} VEGF (Anchordoquy *et al*. 2015). Positive effects of VEGF have also been shown to promote the transition from primary to secondary follicles in the bovine (Yang and Fortune 2007) and to induce follicular growth and increase oocyte diameter in the goat (Bruno *et al.* 2009). In pigs, the presence of 5 ng mL^{-1} VEGF during the IVM of COCs derived from MF significantly increased the blastocyst formation rate and the total number of cells per blastocyst, and reduced the number of apoptotic embryos following parthenogenetic activation (Biswas and Hyun 2011; Kere *et al*. 2014); however, a higher concentration (500 ng mL^{-1}) of VEGF did not have these positive effects (Kere *et al*. 2014). Furthermore, similar effects were observed even if VEGF was only supplemented during the first 20 h of IVM (Kere *et al*. 2014). However, it is not clear whether the presence of VEGF during IVM, especially the first half, is effective in improving the meiotic and developmental competence of oocytes derived from SF.

Therefore, in the present study we compared VEGF content of porcine COCs from MF and SF, as well as that secreted during IVM. In addition, we evaluated the effects of VEGF supplementation during the first 20 h of IVM on improvements in the meiotic and developmental competence of oocytes derived from SF.

Materials and methods

Chemicals and culture media

Sodium chloride, KCl, HCl, NaOH, $MgCl_2 \cdot 6H_2O$, KH_2PO_4, gentamicin sulfate, phenol red and paraffin oil were obtained from Nacalai Tesque, whereas $NaH_2PO_4 \cdot 2H_2O$ and $CaCl_2 \cdot 2H_2O$ were purchased from Ishizu Pharmaceutical and equine chorionic gonadotrophin (eCG; Serotropin) and human chorionic gonadotrophin (hCG; Gonatropin) were purchased from ASKA Pharmaceutical. Unless specified otherwise, all other chemicals were purchased from Sigma-Aldrich.

The medium used for collecting and washing COCs was modified Hepes-buffered Tyrode's Lactate with polyvinyl alcohol (TL-HEPES-PVA), which contained 114 mM NaCl, 3.2 mM KCl, 2 mM $NaHCO_3$, 0.34 mM KH_2PO_4, 10 mM Na-lactate, 0.5 mM $MgCl_2 \cdot 6H_2O$, 2 mM $CaCl_2 \cdot 2H_2O$, 10 mM HEPES, 0.2 mM Na-pyruvate, 12 mM sorbitol, 0.1% (w/v) polyvinyl alcohol, 25 μg mL^{-1} gentamicin and 65 μg mL^{-1} potassium penicillin G. The basic IVM medium used was a bovine serum albumin (BSA)-free chemically defined medium (Porcine Oocyte Medium (POM); Research Institute for the Functional Peptides) supplemented with 50 μM β-mercaptoethanol (mPOM; Akaki *et al*. 2009). The medium used for IVF was Medium-199 (Invitrogen), to which 3.05 mM glucose, 2.92 mM hemi-calcium lactate, 0.91 mM Na-pyruvate, 12 mM sorbitol, 75 mg mL^{-1} potassium penicillin G, 25 mg mL^{-1} gentamicin, 5 mM

caffeine sodium benzoate and 4 mg mL^{-1} BSA were added (mM199; Funahashi and Day 1993). All media (except modified TL-HEPES-PVA) were equilibrated at 39°C in an atmosphere of 5% CO_2 in air overnight before use; though mM199 was equilibrated under paraffin oil.

IVM of COCs

Ovaries without any evidence of corpora lutea were collected from slaughtered commercial gilts at a local public abattoir and transported within 1 h to the laboratory at 32–35°C in 0.9% (w/v) NaCl solution containing 75 μg mL^{-1} potassium penicillin G and 50 μg mL^{-1} streptomycin sulfate. After washing three times with the NaCl solution at room temperature, COCs were aspirated from MF (3–6 mm diameter) and SF (0.5–2 mm diameter) using a 10-mL disposable syringe and 18-gauge needle, placed in 50-mL centrifuge tubes (SF and MF separately) and then washed three times with TL-HEPES-PVA. Only COCs with at least three layers of unexpanded cumulus cells were washed three times with mPOM. Forty COCs each from MF and SF were cultured separately in 500 μL mPOM supplemented with gonadotropins (10 IU mL^{-1} eCG, 10 IU mL^{-1} hCG) and 1 mM dibutyryl cAMP (db-cAMP) in a four-well culture plate (Thermo Fisher Scientific) for 20 h under an atmosphere of 5% CO_2 in air at 39°C (Funahashi *et al*. 1997; Akaki *et al*. 2009). The COCs were then washed three times with fresh IVM medium without gonadotropins and db-cAMP and cultured continuously in the medium for an additional 24 h.

Evaluation of meiotic stage

After IVM, cumulus cells surrounding the oocytes were removed by pipetting in a modified TL-HEPES-PVA medium containing 0.1% (w/v) hyaluronidase (H3506; Sigma). Some of the denuded oocytes were used for IVF and parthenogenetic activation, whereas others were mounted on glass slides and fixed in acetic alcohol (25% (v/v) acetic acid in ethanol) for 2–3 days. The oocytes on glass slides were then stained with 1% (w/v) orcein in 45% (v/v) acetic acid for 5 min and meiotic progression evaluated under a phase contrast microscope at magnifications of ×200 and ×400.

Preparation of fresh boar spermatozoa and IVF

Semen-rich fractions (40–50 mL) collected from a Berkshire boar by the gloved-hand method were donated from a local AI center and processed as reported previously (Funahashi 2005). Briefly, spermatozoa were resuspended (to 1×10^8 cells mL^{-1}) in fresh modified Modena solution containing 5 mM cysteine and 20% (v/v) boar seminal plasma and kept at 15°C until used in IVF (within 2 days of collection). Stored spermatozoa were kept at room temperature for 15–20 min before use, washed three times with Modena solution and then resuspended (to 1×10^8 cells mL^{-1}) in fertilisation medium.

After dilution of the sperm suspension to 1×10^6 cells mL^{-1} with caffeine-free mM199, 50 μL diluted suspension was added to a 50-μL droplet of mM199 containing 10 mM sodium benzoate caffeine and 30–

40 denuded mature oocytes (Wang *et al*. 1991). The oocytes were cocultured with spermatozoa in the 100-μL droplets under paraffin oil at 39°C and an atmosphere of 5% CO_2 in air for 7 h. After washing three times with mM199 supplemented with 0.4% (w/v) BSA, oocytes were cultured in a 50-μL droplet of the same medium under the same conditions for a further 4 h.

Sperm penetration and pronuclear formation assessment

To assess sperm penetration and pronuclear formation, oocytes were washed with TL-HEPES-PVA, mounted on glass slides, fixed in 25% (v/v) acetic acid–ethanol for 2–3 days, stained with 1% (w/v) orcein in 45% (v/v) acetic acid for 5 min and then examined under a phase contrast microscope at magnifications of ×200 and ×400. Oocytes were designated as 'penetrated' when they had at least one sperm head or male pronucleus with corresponding sperm tail in their ooplasm. Oocytes with more than one sperm nuclei or male pronuclei were considered polyspermic.

Parthenogenetic activation and in vitro *culture of oocytes*

Only mature oocytes with the first polar body in the perivitelline space were selected. These oocytes were washed three times with a solution of 0.25 M mannitol containing 0.01% (w/v) PVA, 0.5 mM HEPES, 100 μM $CaCl_2 \cdot H_2O$ and 100 μM $MgCl_2 \cdot 6H_2O$, pH 7.2, then transferred to sit between electrodes separated by a distance of 1 mm in an activation chamber and overlaid with the same solution. A single electrical pulse (direct current; 1.2 kV cm^{-1}, 30 μs) was applied to oocytes to activate them using a BTX Electro-Cell Manipulator 2001M. The activated oocytes were washed three times with mM199 supplemented with 0.4% BSA and 5 μM cytochalasin B and incubated in the same medium at 39°C under an atmosphere of 5% CO_2 in air for 4 h. The oocytes were then washed three times with porcine zygote medium (PZM; Yoshioka *et al*. 2008) and cultured in 500 μL PZM under paraffin oil at 39°C and an atmosphere of 5% CO_2 in air for 5 days. To assess the developmental competence of oocytes derived from MF and SF, cleavage and blastocyst formation rates were observed at 2 and 5 days after the start of culture respectively. Blastocysts were fixed in 4% paraformaldehyde for 15 min at room temperature and then stained with Hoechst 33342 (20 μg mL^{-1} in phosphate-buffered saline (PBS) containing 1% (v/v) Triton X-100) for 30 min at room temperature. Samples were mounted on glass slides and the number of cells per blastocyst counted under a fluorescence microscope (ECLIPSE 80i; Nikon).

VEGF secretion from COCs during IVM

At 20 and 44 h after the start of IVM, the media from 40 COCs in culture were collected into microtubes and stored at –80°C until analysis. The amount of VEGF secreted from COCs into the culture medium was measured using a Quantikine ELISA Human VEGF immunoassay kit (SVE00; R&D Systems) and a microplate reader (Bio-Rad), as described previously by Barboni *et al*. (2000), who

demonstrated that there was no difference between the ability of this system to detect human and pig VEGF.

Experimental design

Experiment 1: VEGF secretion by MF- or SF-derived COCs during IVM and oocyte meiotic competence

Forty COCs derived from MF and SF were cultured according to a standard IVM protocol (as described above) for a total of 44 h. After culture, the meiotic progression of the oocytes was assessed as described above. Furthermore, ELISA was used to determine the amount of VEGF secreted into the IVM media collected 20 and 44 h after the start of IVM. This experiment was replicated six times.

Experiment 2: effects of VEGF supplementation during the first 20 h of IVM on the meiotic competence of SF-derived oocytes

COCs derived from SF were exposed to different concentrations of VEGF (V4512; Sigma-Aldrich; 0, 20, 50, 100 and 200 ng mL^{-1}) for the first 20 h of IVM. After IVM, the meiotic stage of oocytes was evaluated, as described above. This experiment was replicated five times.

Experiment 3: effects of VEGF supplementation during the first 20 h of IVM on the fertilisability of SF-derived oocytes

COCs derived from SF were exposed to 0, 100 and 200 ng mL^{-1} VEGF for the first 20 h of IVM. After IVM culture for a total of 44 h, oocytes were inseminated and cocultured with spermatozoa for 7 h. At 11 h after insemination, oocytes were mounted, fixed and stained with 1% (w/v) orcein in 45% (v/v) acetic acid. The meiotic stage of these oocytes was compared with that of oocytes derived from MF and cultured in the absence of VEGF. This experiment was replicated five times.

Experiment 4: effects of VEGF supplementation during the first 20 h of IVM on the developmental competence of SF-derived oocytes

To determine the developmental competence of oocytes exposed to different concentrations of VEGF (0, 100 and 200 ng mL^{-1}) during the first 20 h of IVM, after a total of 44 h in culture only oocytes with the first polar body were parthenogenetically activated by an electrical pulse and cultured as described above. Cleavage and blastocyst formation rates at 2 and 5 days after the start of culture respectively were compared with those of oocytes derived from MF (positive control); in addition, cell numbers in blastocysts were compared. This experiment was replicated five times.

Statistical analysis

Data from five or six replicated trials were evaluated using one- or two-way analysis of variance (ANOVA) in StatView (Abacus Concepts). To fit a normal distribution, percentage data were arc-sine

transformed before analysis if the data contained percentages >90% or <10%. The variables examined in Experiment 1 were the meiotic stage of oocytes and the amount of VEGF (two-way ANOVA; COCs origin × time interaction when VEGF concentration was measured). In Experiment 2, the meiotic stage of oocytes was evaluated; in Experiment 3, the rate of penetration, monospermy, the formation of male and female pronuclei and the number of spermatozoa per penetrated oocyte were evaluated; finally, in Experiment 4, cleavage rate, blastocyst formation rate and the number of cells in a blastocyst were evaluated. All data are expressed as the mean ± s.e.m. Findings were considered significantly different at $P < 0.05$ and, when there was a significant effect, values were compared with a Dunn–Bonferroni post hoc test.

Results

Experiment 1: VEGF secreted by MF- or SF-derived COCs and meiotic competence of oocytes

The effect of the origin of COCs (MF vs SF) on the resumption of meiosis was examined in 468 oocytes (n=6 for each group). As indicated in Table 1, there were significant differences in the percentage of oocytes at MI and MII between the two groups ($P < 0.01$). The percentage of mature oocytes was significantly higher when COCs were collected from MF than SF.

Conditioned media were collected at 20 and 44 h after the onset of IVM of COCs derived from MF or SF and the amount of VEGF secreted into the media was determined ($n = 6$ replicates). VEGF concentrations secreted into the medium after 20 and 44 h IVM was significantly ($P < 0.001$) greater for COCs derived from MF (115.1 ± 21.2 and 376.9 ± 78.9 pg mL^{-1} respectively) than for those derived from SF (35.6 ± 4.0 and 67.8 ± 16.1 pg mL^{-1} respectively; Table 2). The amount of VEGF collected at the end of the second half of IVM (over a 24- period) was also significantly higher ($P < 0.007$) than that collected at the end of the first half of IVM (20 h), and was nearly doubled.

Experiment 2: effects of VEGF supplementation during the first 20 h of IVM on the meiotic competence of SF-derived oocytes

As shown in Fig. 1, when COCs were exposed to 100 and 200 ng mL^{-1} VEGF during the first 20 h of IVM, the percentages of mature oocytes was significantly higher than in the control group, cultured without VEGF ($P < 0.01$). The higher percentage of mature oocytes was similar to that seen for oocytes from MF-derived COCs cultured in the absence of VEGF supplementation. There were no significant differences in the percentage of mature oocytes when COCs were exposed to 0, 20 or 50 ng mL^{-1} VEGF.

Experiment 3: effects of VEGF supplement during the first 20 h of IVM on the fertilisability of SF-derived oocytes

The percentage of oocytes penetrated and those that formed male and female pronuclei was significantly higher ($P < 0.05$) following exposure of SF-derived COCs to 200 compared with 0 ng mL^{-1} VEGF during the first 20 h of IVM ($n = 5$ replicates; Table 3). There were no significant differences among experimental groups in either the incidence of monospermic penetration, which ranged between 24.5% and 41.5%, and the number of spermatozoa in penetrated oocyte, which ranged between 2.0 and 2.8 (Table 3).

Experiment 4: effects of VEGF supplementation during the first 20 h of IVM on the developmental competence of SF-derived oocytes

The developmental competence of SF-derived oocytes cultured in the presence of 0, 100 or 200 ng mL^{-1} VEGF during the first 20 h of IVM was examined following electrical activation. As indicated in Table 4, data from six replicates demonstrated that supplementation of the IVM medium with 200 ng mL^{-1} VEGF during the first 20 h of IVM significantly improved the rate of blastocyst formation for mature SF-derived oocytes following parthenogenetic activation compared with control. The blastocyst formation rate did not differ significantly different from that of MF-derived oocytes (Table 4).

Discussion

The focus of the present study was on the effects of VEGF on the meiotic ability to reach to the MII stage, fertilisability and developmental competence of oocytes derived from SF. In Experiment1, comparing the meiotic competence of oocytes derived from MF and SF, the number of oocytes maturing to MII was significantly lower in the SF than MF group. With regard to follicular size, many studies in humans (Wittmaack *et al*. 1994), porcine (Marchal *et al*. 2002), bovine (Lonergan *et al*. 1994), goat (Crozet *et al*. 1995), sheep (Cognie *et al*. 1998), dromedary (Khatir *et al*. 2007), buffalo (Raghu *et al*. 2002) and canine (Otoi *et al*. 2000) have demonstrated a clear relationship between follicle size and IVM and fertilisation rates. In the present study, we observed that only half the SF-derived oocytes were fully competent to mature to the MII stage.

The expression of *VEGF* mRNA in granulosa and theca cells is known to increase significantly and is correlated with follicular growth (Berisha *et al*. 2000; Shimizu *et al*. 2003). Intrafollicular concentrations of VEGF have also been demonstrated to be lowest in follicle fluid aspirated from SF and to increase gradually with increases in follicular diameter (Mattioli *et al*. 2001; Kere *et al*. 2014). In the present study, we hypothesised that the lower meiotic competence of oocytes from SF may be affected by the lower concentration of VEGF secreted from COCs into the IVM medium. In Experiment 2, we found that the concentrations of VEGF secreted into the medium at both 20 and 44 h after the start of IVM were

significantly higher when COCs were collected from MF rather than SF. These results are consistent with previous observations in which VEGF content in the follicular fluid was compared among follicles of different sizes (Mattioli *et al*. 2001; Kere *et al*. 2014).

A study with porcine COCs derived from MF has demonstrated that maturation, fertilisation and even blastocyst formation are significantly improved when VEGF was added to the medium during the first 20 h of IVM (Kere *et al*. 2014). However, a high concentration of VEGF (500 ng mL^{-1}) did not affect oocyte competence (Einspanier *et al*. 2002; Biswas *et al*. 2011; Kere *et al*. 2014). In the present study, when SF-derived COCs were exposed to different concentrations of VEGF during the first 20 h of IVM, supplementation with 100 and 200 ng mL^{-1} VEGF significantly improved the number of oocytes at MII to levels similar to those seen for oocytes from MF. These results suggest that the acquisition of meiotic competence by oocytes is achieved in the presence of VEGF at appropriate concentrations during the first 20 h of IVM, even when the COCs are obtained from SF. The effective concentrations of VEGF appear to be 100 and 200 ng mL^{-1} for COCs derived from SF, but may be different for those COCs from MF. Previous studies have indicated that VEGF can protect bovine granulosa cells from apoptotic cell death and follicle atresia (Greenaway *et al*. 2004; Kosaka *et al*. 2007). It is well known that VEGF induces activation of the mitogen-activated protein kinase kinase/extracellular signal-regulated kinase pathway via VEGF receptor 2 (VEGF-R2), preventing cells from undergoing atresia (Wang *et al*. 2009). Considerable evidence indicates that VEGF suppresses damage to cumulus cells (Shin *et al*. 2006) and enhances cumulus cell expansion *in vitro* (Biswas *et al*. 2010). Therefore, VEGF may play a key role preventing the apoptosis of cumulus cells, indirectly participating in maintaining the meiotic competence of oocytes. So it is possible that a decrease in the amount of VEGF secreted by COCs may be one causes for the decreased competence of SF-derived oocytes.

Furthermore, when matured oocytes were subjected to IVF, the percentage of oocytes penetrated and those forming a male pronucleus was significantly higher for SF-derived COCs exposed to 200 ng mL^{-1} VEGF during the first 20 h of IVM, with values similar to those fro MF-derived oocytes. The fertilisability of oocytes, including the competence to form male and female pronuclei after sperm penetration, is considered one of the indicators of oocyte cytoplasmic maturation (Watson 2007). In the present study, in Experiment 5, we examined developmental competence following parthenogenetic activation because a high incidence of polyspermic penetration following IVF of IVM porcine oocytes has frequently been reported (Funahashi 2003; Romar *et al*. 2016). Herein we demonstrated that the developmental competence of SF-derived oocytes was significantly improved when the COCs were supplemented with 200 ng mL^{-1} VEGF during the first 20 h of IVM. Recently, it was reported that the addition of 5 ng mL^{-1} VEGF to the IVM medium improved the quality of mature porcine oocytes derived

from MF, as well as developmental competence following parthenogenetic activation (Kere *et al*. 2014). The results of the present study indicate that VEGF is effective in improving both the quality and developmental competence of oocytes, even when derived from SF, although the appropriate concentration appears to differ between COCs derived from MF and SF. Therefore, the presence of 200 ng mL^{-1} VEGF during the first 20 h period of IVM appears to improve the abilities of SF-derived oocytes to develop to the MII and blastocyst stages following IVM and IVC, respectively. Supplementation of the IVM medium with VEGF will make it possible to use COCs not only from MF, but also from SF for the *in vitro* production of porcine embryos.

In conclusion, COCs derived from SF secrete lower concentrations of VEGF than those from MF. Supplementation of the IVM medium with 200 ng mL^{-1} VEGF during the first 20 h of IVM improved the meiotic ability to the MII stage, fertilisability and developmental competence of SF-derived oocytes. Therefore, we recommend the addition of 200 ng mL^{-1} VEGF during the first 20 h of IVM to optimise results of *in vitro* embryo production from COCs derived from SF.

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Fig. 1. Effect of vascular endothelial growth factor (VEGF) supplementation during the first 20 h of IVM on the meiotic competence of oocytes (% of mature ones) derived from medium-sized follicles (MF; 3–6 mm diameter) and small follicles (SF; 0.5–3 mm diameter). Data are the mean ± s.e.m. **P* < 0.05 compared with MF (*n* = 5 replicates).

Table 1. Meiotic progression of oocytes derived from medium-sized follicles (MF; 3–6 mm diameter) and small follicles (SF; 0.5–3 mm diameter) 44 h after the start of IVM

Data are the mean ± s.e.m. (*n* = 6 replicates for each group). Within columns, values with different superscript letters differ significantly (*P* < 0.05). COCs, cumulus–oocyte complexes; GV, germinal vesicle; AI/TI: anaphase I/telophase I

Origin of COCs	No. oocytes examined	% Oocytes				
		Degenerated	GV	MI	AI/TI	MII
MF	230	2.3 ± 1.0	2.5 ± 1.6	16.3 ± 1.9[a]	0.5 ± 0.5	78.4 ± 0.7[a]
SF	238	8.1 ± 2.8	3.8 ± 2.4	36.7 ± 7.3[b]	0.8 ± 0.8	50.6 ± 1.5[b]

Table 2. Concentration of vascular endothelial growth factor (VEGF) secreted from cumulus–oocyte complexes (COCs) from medium-sized follicles (MF; 3–6 mm diameter) and small follicles (SF; 0.5–3 mm diameter) into the medium 20 and 44 h after the start of IVM, as well as the significance of the origin of COCs and the duration of IVM on VEGF concentration in the medium

Data are the mean ± s.e.m. Within columns, values with different superscript letters differ significantly ($P < 0.05$)

	VEGF (pg mL^{-1})	
	20 h IVM	44 h IVM
COC origin		
MF	115.1 ± 21.2[a]	376.9 ± 78.9[a]
SF	35.6 ± 4.0[b]	67.8 ± 16.1[b]
P-values ($n = 6$)		
COC origin	$P < 0.001$	
Duration of IVM	$P < 0.007$	

Table 3. Effect of vascular endothelial growth factor (VEGF) supplementation during the first 20 h of IVM on sperm penetration and pronuclear formation of oocytes

Unless indicated otherwise, data show the mean ± s.e.m. ($n = 5$). Within columns, values with different superscript letters differ significantly ($P < 0.05$). COC, cumulus–oocyte complex; MF, medium-sized follicles (3–6 mm diameter); SF, small follicles (0.5–3 mm diameter)

COC origin	VEGF (ng mL^{-1})	No. oocytes examined	Penetrated		Monospermy		Male and female pronuclei formed		Sperm-penetrated oocytes
			n	%	*n*	%	*n*	%	
MF	0	198	157	79.3 ± 4.1[a]	36	24.5 ± 3.3	150	75.7 ± 3.3[a]	2.8 ± 0.3
SF	0	195	117	60.0 ± 5.6[b]	38	41.5 ± 9.9	97	49.7 ± 3.5[b]	2.0 ± 0.2
SF	100	197	134	68.0 ± 5.9[ab]	33	29.0 ± 1.5	114	57.9.0 ± 2.9[b]	2.8 ± 0.4
SF	200	194	153	78.9 ± 2.0[a]	38	27.9 ± 6.1	137	70.6 ± 1.5[a]	2.8 ± 0.4

Table 4. Effects of vascular endothelial growth factor (VEGF) supplementation during the first 20 h of IVM on the developmental competence of oocytes from small follicles (SF; 0.5–3 mm diameter)

Unless indicated otherwise, data show the mean ± s.e.m. ($n = 6$). Within columns, values with different superscript letters differ significantly ($P < 0.05$). MF, medium-sized follicles (3–6 mm diameter)

Origin of oocytes	VEGF (ng mL^{-1})	No. mature oocytes examined	Cleaved		Blastocysts formed		No. cells per blastocyst
			n	%	*n*	%	
MF	0	184	171	93.0 ± 2.0	52	28.2 ± 1.4[a]	31.7 ± 1.3
SF	0	132	115	87.0 ± 1.0	18	13.6 ± 2.8[b]	29.2 ± 1.0
SF	100	164	142	86.2 ± 2.3	35	21.3 ± 2.9[ab]	29.1 ± 1.4
SF	200	166	150	90.3 ± 1.3	41	24.6 ± 2.4[a]	33.8 ± 3.0