

# Vascular Endothelial Growth Factor Has Beneficial Effect Independent of Serum Components throughout Oocyte Maturation and Early Embryonic Development in Cattle

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**ABSTRACT :** In our previous studies, we demonstrated that Vascular Endothelial Growth Factor (VEGF) enhances bovine oocyte maturation and early embryonic development in serum supplemented media. In this experiment, to determine the synergistic effect of VEGF with serum components on early embryonic development *in vitro* in cattle, 1 mg/ml polyvinyl-alcohol (PVA) was replaced with foetal bovine serum (FBS) in maturation and culture media. Bovine oocytes were matured in Synthetic Oviduct Fluid (SOF) supplemented with PVA, PVA+5 ng/ml of VEGF, FBS, or FBS+VEGF. Fertilized oocytes were cultured in the same conditions for 8 days. The development of embryos was examined at 48 h post- insemination and on days 6, 7 and 8. The results were analyzed using repeated measures two- factor ANOVA, in which the effects of VEGF and serum were assigned as two factors. The development rate to 4- to 8-cell embryos at 48 h was significantly higher in the PVA+VEGF group than in the PVA group (44.7% and 31.5%, respectively). However, the highest development rate to 4- to 8-cell embryos was obtained from the FBS+VEGF group (58.8%). On day 8, the blastocyst rates were higher in the PVA+VEGF (22.8%), FBS (32.1%,  $p < 0.05$ ) and FBS+VEGF (42.1%,  $p < 0.05$ ) groups than in the PVA group (17.1%). Two- factor ANOVA of the development rates indicates that VEGF had a significant effect, but had no synergistic effect with serum components on early embryonic development. The results of the present study demonstrate that VEGF improves the *in vitro* developmental competence of bovine oocytes and/or embryos independent of the effect of serum components. (*Asian-Aust. J. Anim. Sci.* 2006. Vol 19, No. 4 : 495-499)

**Key Words :** Bovine Embryo, Early Development, IVM-IVF-IVC, Serum, VEGF

## INTRODUCTION

Within the mammalian reproductive system, the oviduct provides an optimal microenvironment for the development of zygote. The composition and the amount of bovine oviductal fluid change depending on the stage of estrous cycle, with more fluid at estrus and ovulation (Ehrenwald et al., 1990; Gerena et al., 1990). Various proteins and growth factors secreted into the oviduct influence sperm capacitation, final oocyte-maturation, fertilization, and early embryonic development (Gandolfi et al., 1991, 1993; Heyner et al., 1993). However the expression of many growth promoting factors, such as Fibroblast Growth Factor (FGF), Epidermal Growth Factor (EGF), Transforming Growth Factor (TGF)  $\beta$ 1, Vascular Endothelial Growth Factor (VEGF) and so on (Gerena et al., 1990) differs in animal species (Heyner et al., 1993).

VEGF is known as a potent mitogen for micro- and macro-vascular endothelial cells (Leung et al., 1989;

Ferrara, et al., 1997) and as a stimulator of vascular permeability based on its ability to induce vascular leakage (Connolly et al., 1989, 1989, 1991).

Gabler et al. (1989, 1996) suggested that VEGF is involved in creating an optimal local environment for fertilization and/or early embryonic development by modulating permeability in the bovine oviduct. Einspanier et al. (1997) found that VEGF transcripts increased continuously in bovine granulosa cells according to follicular development and its concentration in follicular fluid was 5-fold higher in preovulatory follicles (5 ng/ml) than in early antral follicles (1 ng/ml). These findings suggested that VEGF be involved in maturation of oocyte or the early development of embryo in cattle. We accordingly presumed that VEGF affects on oocyte maturation *in vitro*, and have shown that VEGF has beneficial effects on bovine oocyte maturation and early embryonic development in serum-supplemented media (Luo et al., 2002a, b).

The objectives of this study were to determine the effect of VEGF on the *in vitro* developmental competence of bovine oocytes and embryos in serum-free condition and the synergistic effect of VEGF and serum component.

## MATERIALS AND METHODS

### Reagents

Recombinant human VEGF<sub>165</sub>, obtained from R & D

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**Table 1.** Experimental design to analyze the synergistic effect of VEGF with serum components on the *in vitro* development of bovine embryos

Treatment*	IVM	IVC1**	IVC2**
PVA	PVA	PVA	PVA
PVA+VEGF	PVA+VEGF	PVA+VEGF	PVA+VEGF
FBS	FBS (10%)	FBS (1%)	FBS (5%)
FBS+VEGF	FBS (10%)+VEGF	FBS (1%)+VEGF	FBS (5%)+VEGF

PVA: Polyvinyl-alcohol; VEGF: Vascular endothelial growth factor; FBS: Fetal bovine serum.

IVM: *In vitro* maturation; IVC: *In vitro* culture.

\* PVA: 1 mg/ml; VEGF: 5 ng/ml.

\*\* IVC1 and 2: IVC until 48 h Pi and IVC from 48 h Pi, respectively.

**Table 2.** Effect of VEGF on the development of bovine embryos cultured in serum-free and serum-supplemented media

Treatment*	No. of replicates	No. of oocytes	No. (%±SE) of oocytes developed to			No. (%±SE) of 4- to 8-cells developed to blastocyst on day 8
			≥2 cell**	4- to 8-cell**	Blastocyst on day 8	
PVA	6	111	49 (44.1±4.3) <sup>a</sup>	35 (31.5±5.5) <sup>a</sup>	19 (17.1±6.2) <sup>a</sup>	19 (44.0±16.2) <sup>a</sup>
PVA+VEGF	6	114	62 (54.5±1.8) <sup>b</sup>	51 (44.7±2.5) <sup>b</sup>	26 (22.8±3.9) <sup>a</sup>	26 (50.3±8.0) <sup>a</sup>
FBS	6	112	66 (58.8±3.2) <sup>b</sup>	56 (50.0±3.7) <sup>bc</sup>	36 (32.1±1.9) <sup>b</sup>	36 (65.6±5.8) <sup>a</sup>
FBS+VEGF	6	114	79 (69.3±4.0) <sup>c</sup>	67 (58.8±4.0) <sup>c</sup>	48 (42.1±1.8) <sup>c</sup>	48 (72.5±4.4) <sup>a</sup>

PVA: Polyvinyl-alcohol; VEGF: Vascular endothelial growth factor; FBS: Fetal bovine serum.

\* PVA: 1 mg/ml; VEGF: 5 ng/ml.

\*\* at 48 h Pi.

<sup>a, b, c</sup> Values in the same column with different superscripts differ significantly ( $p < 0.05$ ).

Systems (Minneapolis, MN), was employed at 5 ng/ml. SOF was supplemented with 1% BME-EAA (essential amino acid, Sigma B-6766, St. Louis, MO, USA), 1% MEM-NEAA (non-essential amino acid, MEM-NEAA, Sigma M-7145), 1.5 mM Glucose (Dextrose anhydrous, Wako Pure Chemical Industries, Osaka, Japan), 0.5 mM pyruvic acid (Sigma P-4562), 100 IU/ml penicillin (Sigma Pen-K), 100 µg/ml streptomycin (Meiji Seika, Tokyo, Japan) and 1 mg/ml polyvinyl-alcohol (PVA, Sigma P-8136) or 10% fetal bovine serum (FBS, ICN Biomedical Inc., 29-167-54, Aurora, OH, USA) for *in vitro* maturation (IVM), and 1 mg/ml PVA or 1% FBS for *in vitro* culture (IVC). For *in vitro* fertilization (IVF), BO medium (Brackett and Oliphant, 1975) was supplemented with 20 µg/ml heparin (Sigma H-3149) and 3 mg/ml fatty acid-free bovine serum albumin (BSA, Sigma A-7511).

#### *In vitro* maturation (IVM)

Bovine ovaries were obtained at a local abattoir and transported to the laboratory in physiological saline (30–35°C) within 5 h. Follicular contents were aspirated from small antral follicles (2–5 mm) using a 20-gauge needle attached to a 10 ml disposable syringe, then allowed to settle in a Petri dish and the supernatant was discarded. Cumulus oocyte complexes (COC) with multilayered compact cumulus cells were selected for maturation *in vitro*. Groups of 20±2 COC were matured in 100 µl droplets of SOF supplemented with 2 g/ml porcine Follicle Stimulating Hormone (FSH, Antrin, Denka Pharmaceutical, Kawasaki, Japan), 2 g/ml Estradiol-17 (Sigma E-1127) and 10% FBS

or 1 mg/ml PVA under paraffin oil for 22 h at 39°C in an atmosphere of 5% CO<sub>2</sub> in air.

#### *In vitro* fertilization (IVF)

Matured COC were washed 3 to 4 times with the BO medium and transferred each 20±2 to 100 µl fertilization droplets of the same medium under paraffin oil. For the capacitation of spermatozoa, frozen-thawed (37°C) Japanese Black semen was layered on 45 and 60% discontinuous Percoll (Amersham Pharmacia Biotech, Piscataway, NJ, USA) gradient BO medium and centrifuged 15 min at 800×g, 37°C. The sperms were diluted at a final concentration of 5×10<sup>6</sup> sperms/ml. Gametes were incubated together 6 h in the BO medium under paraffin oil at 39°C in an atmosphere of 5% CO<sub>2</sub> in air.

#### *In vitro* culture (IVC)

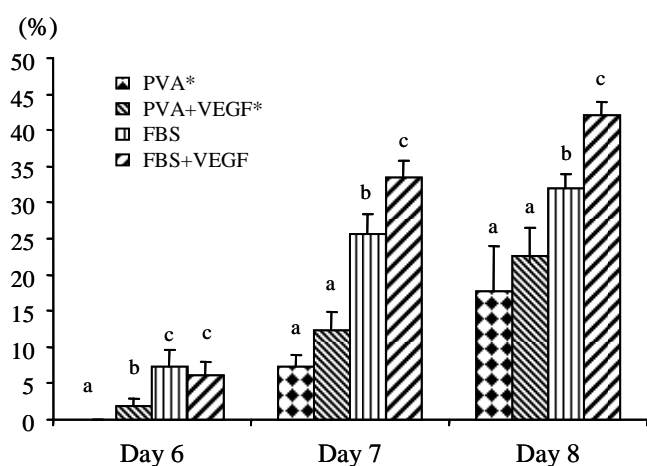
The presumptive embryos were washed 3 to 4 times with SOF supplemented with 1% FBS or 1 mg/ml PVA at 6 h post-insemination (Pi), then placed each 20±2 in 100 µl droplets of the same medium under paraffin oil and cultured at 39°C in an atmosphere of 5% CO<sub>2</sub>, 5% O<sub>2</sub>, 90% N<sub>2</sub> until 48 h Pi. Then, cumulus cells surrounding embryos were removed gently with a suitable glass pipette. The denuded embryos were washed 3 to 4 times with SOF supplemented with 5% FBS or 1 mg/ml PVA, then, transferred each 20±2 to 30 µl droplets of the same medium under paraffin oil and cultured under the same condition as the preceding culture for 120 h.

**Table 3.** The results (P values) in the statistical analysis by repeated measures two-factor ANOVA on the effect of VEGF and serum

Factors*	CR	IDR	M6	B6	B7	B8	B8 from 4- to 8-cell
VEGF	<0.005	<0.02	<0.02	0.32	<0.02	<0.01	0.51
FBS	<0.0001	<0.001	0.43	<0.0001	<0.0001	<0.001	<0.05
F×V	0.85	0.62	0.40	0.92	0.59	0.51	0.97

PVA: Polyvinyl-alcohol; VEGF: Vascular endothelial growth factor; FBS: Fetal bovine serum; CR: Cleavage rate; IDR: Initial development rate (Number of 4- to 8-cell embryos/number of oocytes at 48 h Pi); M6: Number of morulas/number of oocytes on day 6; B6, B7, B8: Number of blastocysts/number of oocytes on days 6, 7 and 8, respectively. B8 from 4- to 8-cell: Number of blastocysts on day 8/number of 4- to 8-cell embryos at 48 h Pi.

\* VEGF: Effect of VEGF; FBS: Effect of FBS; F×V: Synergistic effect of VEGF and FBS added to synthetic oviduct fluid (SOF).



**Figure 1.** Blastocyst rates on days 6, 7 and 8. PVA: Polyvinyl-alcohol; VEGF: Vascular endothelial growth factor; FBS: fetal bovine serum. \* PVA: 1 mg/ml; VEGF: 5 ng/ml. <sup>a, b, c</sup> Values on the shoulder of bars with different superscripts differ significantly ( $p < 0.05$ ).

The design of the experiment is shown in Table 1. Cleavage rates and development rates to 4 to 8-cell stage were assessed at 48 h Pi. Development rates to blastocyst were examined on days 6 (144 h Pi), 7 (168 h Pi) and 8 (192 h Pi).

### Statistical analysis

Data were presented as percentages or means with standard errors. Repeated measures two-factor ANOVA was carried out to examine the synergistic effect of VEGF and serum components. Differences were considered to be significant at  $p < 0.05$ .

## RESULTS

The results in the experiment are summarized in Table 2 and Figure 1. Table 3 shows the results (P values) in the statistical analysis by repeated measures two-factor ANOVA of the effect of VEGF and serum on the early development of bovine embryos. As shown in Table 2, the cleavage and the development rates to 4- to 8-cell stage were significantly higher ( $p < 0.05$ ) in the PVA+VEGF group than those in the PVA group. However, the highest cleavage and development rates to blastocyst from oocyte and from

4- to 8-cell stage were obtained from the FBS+VEGF group. As shown in Table 3, both VEGF and FBS have significant effects on the development of fertilized bovine oocytes but have no synergistic effect on the development. Although VEGF showed no significant effect on the development of 4- to 8-cell embryos to blastocysts (Table 3), FBS was shown to have the effect of promoting the development of 4- to 8-cell embryos ( $p < 0.05$ ).

In Figure 1, on day 6, early blastocysts were observed in the PVA+VEGF group, but not in the PVA group. However, mid and late stage blastocysts were observed on day 6 in the FBS and FBS+VEGF groups, respectively. In the PVA group, first early blastocyst was observed on day 7. On day 8, the development rates to blastocyst were higher ( $p < 0.05$ ) in the PVA+VEGF, FBS and FBS+VEGF groups than in the PVA group. Although, in serum-supplemented media, the effect of VEGF on the development of embryos *in vitro* was greater than in serum-free media, the synergistic effects between VEGF with serum components was not significant ( $p > 0.05$ ) as shown in Table 3. These results indicate that VEGF has beneficial effects on the developmental competence of bovine oocytes and/or embryos independent of the effect of serum components.

## DISCUSSION

The rates of the first polar body formation and fertilization of *in vitro*-matured oocytes were lower than those of oocytes matured *in vivo* (Trounson et al., 1977). This might be the result of incomplete cytoplasmic maturation *in vitro* (Gandolfi et al., 1993). Increasing numbers of investigations have dealt with the effect of growth factors in mammalian oocytes and embryos (Gandolfi et al., 1991; Larson et al., 1992; Heyner et al., 1993; Host-Hansen et al., 1993; Keefer et al., 1994; Im and Park, 1995; Lee et al., 1995; Park et al., 1997; Kita and Okumura, 2001).

In our previous studies (Luo et al., 2002a, b), we demonstrated that VEGF increases the maturation and the normal fertilization rates of bovine oocytes, and the development rates to blastocyst *in vitro*. These results indicate that VEGF added to maturation and culture media enhances the developmental competence of mammalian oocytes and embryos. Furthermore, it was suggested that

VEGF be an important factor not only for nuclear maturation but also for cytoplasmic maturation of bovine follicular oocyte. However, VEGF has no effect on the development of bovine embryo without cumulus cells.

As shown in Table 2, VEGF did not increase the development rate from 4- to 8- cell embryos to blastocysts in serum- free media, the same as in serum supplemented-media. These results are consistent with our previous report (Luo et al., 2002b). So, it implies that the promoting effect of VEGF on the development of bovine embryos comes out through cumulus cells, similar to the suggestion of Im et al. (Im and Park, et al., 1995) on EGF. In our hypothesis, VEGF acts via a cAMP- dependent process in the cumulus cell, as it plays a role in angiogenesis. The role of VEGF may be subtle in cytoplasmic maturation, evincing its positive effect in early event related embryogenesis.

Figure 1 showed that the development rates to blastocyst on day 6 were significantly higher in the PVA+VEGF, FBS and FBS+VEGF groups than in the PVA group, respectively. In the PVA group, first early blastocyst was observed on day 7. The development speed of embryos until day 6 was greater in order of the FBS+VEGF, FBS, PVA+VEGF and PVA groups. The results on days 7 and 8 were consistent with those on day 6. These results indicate that VEGF has a beneficial effect on the early development of bovine embryos in serum-free media as well as in serum-supplemented media. However, serum supplementation more enhanced the early development of bovine embryos than VEGF supplementation. As shown in Table 3, VEGF had no effect on cumulus free embryos after 48 h Pi, whereas serum still showed significant effect on those stage embryos. This is thought to be the cause of the difference of development rates to blastocyst between VEGF and serum supplementations.

The maturation of oocytes and the development of embryos occurs in response to an ever-changing milieu of gonadotropins, growth factors, steroids, factors secreted by the oocytes and the embryos themselves, and other unknown molecules, such as in serum (Connolly, 1991).

There may be synergistic effects of growth factors on oocyte maturation and embryonic development. Some studies on synergistic effects of growth factors have been attempted in bovine embryos, such as platelet derived growth factor (PDGF) and insulin-like growth factor-I (IGF-I) (Larson et al., 1992; Kita and Okumura, 2001), EGF and TGF  $\beta$ 1 (Keefer et al., 1994), FGF and EGF (Lee and Fuku, 1995), TGF  $\beta$ 1 and FGF (Larson et al., 1992). Gupta et al. (2005) reported that TCM 199 supplemented with 20 ng/ml of EGF and steer serum stimulated the cumulus cells expansion and increased *in vitro* maturation rate of oocytes in buffaloes. However, to date, very little is known about synergistic effects of VEGF on the bovine

oocyte maturation and embryonic development. In our experiments, the synergistic effects between VEGF and serum components were significant neither on maturation of bovine oocytes nor on the early development of its embryos. These results showed that the effects of VEGF on maturation and early development are independent of the effect of serum components. It is accordingly suggested that those two factors have different mechanisms to promote oocyte maturation and early embryonic development in cattle.

In conclusion, the results of this experiment clearly demonstrated that VEGF improves the developmental competence of bovine oocyte and/or embryo both in serum-supplemented and serum- free media, and the effects of VEGF on bovine oocyte and embryo are independent of serum components. However, it is still not clear how VEGF acts on oocyte maturation and embryonic development *in vitro* as well as *in vivo* and further study is necessary for elucidation.

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