FULL PAPER *Theriogenology*

Effect of Vascular Endothelial Growth Factor on Maturation, Fertilization and Developmental Competence of Bovine Oocytes

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ABSTRACT. To examine the effect of Vascular Endothelial Growth Factor (VEGF) on the maturation of bovine oocytes, human recombinant $VEGF_{165}$ was used in 3 experiments. In Exp. 1, bovine cumulus oocyte complexes (COCs) were matured for 22 hr in modified Synthetic Oviduct Fluid (m-SOF) supplemented with 0 (control) or 5 *n*g/m*l* of VEGF. Maturation rate increased ($P<0.05$) from 78.2% in the control to 90.5% in the VEGF treated group. In Exp. 2, bovine COCs were matured in m-SOF and co-incubated with sperm in modified BO medium, each supplemented with or without 5 *n*g/m*l* VEGF. Normal fertilization rate was improved ($P<0.05$) from 63.0% (control) to 79.8% or 82.3% with VEGF during maturation or both maturation and fertilization. In Exp. 3, bovine COCs were matured the same way as in Exp. 1, then co-incubated with sperm for 6 hr and cultured for 162 hr in m-SOF without VEGF. Cleavage rate and development rate to the 4- to 8-cell stage were examined at 42 hr post-co-incubation and development rate to blastocyst was examined at 162 hr post-co-incubation. Cleavage, the development to the 4- to 8-cell stage and blastocyst rates (82.0%, 70.3% and 45.1%, respectively) were significantly higher ($P<0.05$) in the VEGF group than those in the control (67.3%, 52.5% and 33.3%, respectively). These results indicate that VEGF has a beneficial effect on the maturation of bovine oocytes.

KEY WORDS: bovine oocyte, early embryonic development, fertilization, maturation, Vascular Endothelial Growth Factor.

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In the mammalian reproductive system, the oviduct provides an optimal microenvironment for the development of the zygote. Oviductal fluid influences final oocyte-maturation, sperm capacitation, fertilization and early embryonic development. The composition and the amount of fluid produced by the bovine oviduct change according to the stage of the estrous cycle, with more fluid at estrus and ovulation [5, 13]. The oviduct secretes many growth promoting factors, such as Fibroblast Growth Factor (FGF), Epidermal Growth Factor (EGF), Transforming Growth Factor (TGF) β1, Vascular Endothelial Growth Factor (VEGF) and others [12]. Expression of these growth factors differs between animal species [15].

VEGF is known as a potent mitogen for micro- and macro-vascular endothelial cells [7, 17] and as a stimulator of vascular permeability based on its ability to induce vascular leakage [2–4, 20]. It is essential for the development of follicles and corpora lutea in the ovary, and the establishment of vascular structure in the placenta [8]. The expression of VEGF is correlated with vascularization of tissues during the female reproductive cycle including embryogenesis [18, 21].

Gabler *et al.* [10, 11] suggested that VEGF might be involved in creating an optimal local environment for fertilization and/or early embryonic development by modulating permeability in the bovine oviduct. Einspanier *et al.* [6] found that VEGF transcripts increased continuously in bovine granulosa cells accompanied by follicular development and the concentration of VEGF in follicular fluid was 5-fold higher in preovulatory follicles (5 *n*g/m*l*) compared with early antral follicles (1 *n*g/m*l*). These findings suggest that VEGF is involved in the maturation of oocytes or the early development of embryos in cattle. The aim of this study was to investigate the effect of VEGF on maturation of bovine oocytes *in vitro*.

MATERIALS AND METHODS

Reagents: Human recombinant $VEGF_{165}$ derived from Sf21 insect cells was obtained from R&D systems (Minneapolis, MN, U.S.A.) and utilized at 5 *n*g/m*l*. For *in vitro* culture of bovine oocytes and embryos, Synthetic Oviduct Fluid (SOF) [22] was supplemented with 1% basal medium Eagle-essential amino acid (BME-EAA, Sigma B-6766, St. Louis, MO, U.S.A.), 1% minimum essential medium-nonessential amino acid (MEM-NEAA, Sigma M-7145), 5 mM taurine (Sigma T-7146), 0.5 mM pyruvic acid (Sigma P-4562), 100 UI/m*l* penicillin (Sigma Pen-K) and 100 μg/m*l* streptomycin (Meiji Seika, Tokyo, Japan). For *in vitro* fertilization, BO medium [1] was supplemented with 20 μg/m*l* heparin (Sigma H-3149) and 3 mg/m*l* 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 hr. Follicular contents were aspirated from small antral follicles (2–5 mm) using an 20-gauge needle attached to a 10 m*l* disposable syringe, then allowed to settle in a Petri dish and the supernatant was dis-

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carded. Only cumulus oocyte complexes (COCs) with multilayered compact cumulus cells were selected for maturation *in vitro*. The COCs were washed 3 times with SOF containing 10% Fetal Bovine Serum (FBS, ICN Biomedical Inc., Aurora, OH, U.S.A.), 1.5 mM glucose (Dextrose anhydrous, Wako Pure Chemical Industries, Osaka, Japan), 2 μg/m*l* Follicle Stimulating Hormone (FSH, Antrin, Denka Pharamaceutical, Kawasaki, Japan), 2 μg/m*l* estradiol-17β (Sigma E-1127). Groups of 20 ± 2 COCs were matured in 100 μl droplets of the same medium under paraffin oil for 22 hr at 39°C in an atmosphere of 5% CO_2 in air.

In vitro fertilization (IVF): After IVM, COCs were washed 3 to 4 times with BO medium and transferred in groups of 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, U.S.A.) gradient BO medium and centrifuged 15 min at 800 × g, 37°C. The sperms were diluted to a final concentration of 5×10^6 sperms/m*l*. Gametes were co-incubated for 6 hr in BO medium under paraffin oil at 39°C in an atmosphere of 5% CO_2 in air.

In vitro culture (IVC): The presumptive embryos were washed 3 to 4 times with SOF supplemented with 1% FBS following IVF, then placed 20 ± 2 embryos in a 100 μl droplet of the same medium under paraffin oil and cultured at 39°C in an atmosphere of 5% CO_2, 5% O_2, 90% N_2 until 42 hr post-co-incubation. Then, cumulus cells surrounding the embryos were removed gently by a suitable glass pipette. The denuded embryos were washed 3 to 4 times with SOF supplemented with 5% FBS, then transferred in groups of 20 ± 2 to 30 μl droplet of the same medium under paraffin oil and cultured under the same conditions as the preceding culture for 120 hr.

Exp. 1. Effect of VEGF on maturation of bovine oocytes: The effect of VEGF on maturation was assessed by the maturation rate at 22 hr of IVM in medium supplemented with or without VEGF. The oocyte was fixed for 90 hr in a fixative (glacial acetic acid: ethanol, 1: 3), stained with 1% aceto-orcein (1% orcein in 45% acetic acid), preserved with acetoglycerol solution (glacial acetic acid: glycerin: H_2O, 1: 1: 3) and evaluated microscopically. Oocytes at metaphase-II were considered to have matured.

Exp. 2. Effect of VEGF on fertilization of bovine oocytes: The effect of VEGF on fertilization was assessed by penetration and fertilization rates after 10 hr of co-incubation with sperms. The COCs were matured *in vitro* with or without VEGF, then co-incubated with or without VEGF. The oocytes were fixed for 80 hr, stained, preserved and evaluated microscopically. The oocytes with one male and one female pronuclei and one sperm tail in the ooplasma were considered to have been fertilized normally.

Exp. 3. Effect of VEGF on developmental competence of bovine oocytes: IVM of bovine oocytes was performed with or without VEGF the same as in Exp. 1. IVF and IVC were performed without VEGF. The cleavage rate and development rate at the 4- to 8-cell stage were assessed at 42 hr post-co-incubation. The development rate to the blastocyst was examined at 162 hr post-co-incubation.

Statistical analysis: Data are presented as percentages or means with standard errors. Repeated measures of one-way ANOVA were carried out in Exp. 1 and 3. The results of Exp. 2 were subjected to repeated measures of two-way ANOVA. Differences were considered to be significant at $P<0.05$.

RESULTS

In Exp. 1, a total of 260 bovine oocytes were examined for the maturation rate in 7 replicates. VEGF significantly increased the maturation rate of the oocytes compared with the control (Table 1).

In Exp. 2, 462 oocytes were examined in 7 replicates. As shown in Tables 2 and 3, the maturation, penetration and fertilization rates were significantly increased when the oocytes were matured with VEGF. However, the effect of VEGF supplement during insemination on these rates was not significant ($P>0.05$). There was no significant interaction between groups supplemented with VEGF during maturation and insemination (Table 3).

In Exp. 3, in 6 replicates, 225 oocytes were examined. As shown in Table 4, VEGF added to the maturation medium significantly enhanced the cleavage rate, and the development rates up to the 4- to 8-cell stage and the blastocyst. However, the development rate from the 4- to 8-cell stage to the blastocyst in the group with VEGF was similar ($P>0.05$) to the control.

DISCUSSION

Many published reports have dealt with the effect of growth factors on mammalian oocytes [19], but the effect of VEGF on oocyte maturation has not been reported. The results of the present study demonstrate that VEGF increased ($P<0.05$) the maturation (Exp. 1 and 2), penetration and fertilization rates (Exp. 2) of bovine oocytes. The cleavage, the development rates from the oocyte to the 4- to 8-cell stage and the blastocyst were higher ($P<0.05$) in the group with VEGF than the control (Exp. 3). However, the development rate from the 4- to 8-cell stage embryo to the blastocyst stage were similar ($P>0.05$) in both VEGF and

Table 1. Effect of VEGF on nuclear maturation of bovine oocytes cultured in modified SOF

Treatment	No. of replicates	No. of oocytes examined	No. of matured oocytes (% ± SEM)
–	7	126	98 (78.2 ± 4.7)[a)]
+	7	134	121 (90.5 ± 2.2)[b)]

+: matured with 5 *n*g/m*l* VEGF; –: matured without VEGF.
a,b) Values in the same column with different superscripts differ significantly ($P<0.05$).

Table 2. Effect of VEGF on fertilization of bovine oocytes matured and fertilized *in vitro*

Treatment		No. of	No. of oocytes	No. of oocytes (% ± SEM)		
M	F	replicates	examined	Metaphase-II	penetrated	fertilized normally
–	–	7	120	101 (84.2 ± 2.4)	78 (65.6 ± 5.8)	75 (63.0 ± 6.0)
–	+	7	118	100 (84.6 ± 2.5)	91 (77.0 ± 4.7)	88 (74.5 ± 6.2)
+	–	7	109	99 (90.5 ± 3.1)	89 (81.8 ± 3.7)	87 (79.8 ± 4.5)
+	+	7	115	105 (91.1 ± 1.9)	98 (85.0 ± 2.8)	95 (82.3 ± 3.6)

M: IVM; F: IVF; +: With 5 *n*g/m*l* VEGF; –: Without VEGF.

Table 3. Significance (P value) in statistical analyses of data in Table 2 by two-way ANOVA

Period	Metaphase-II	Penetrated	Fertilized normally
Maturation	**0.02**	**0.01**	**0.03**
Fertilization	0.86	0.11	0.20
Interaction	0.96	0.34	0.41

Table 4. Effect of VEGF on the developmental competence of bovine embryos produced *in vitro**

Treatment	No.of	No.of oocytes	No. (% ± SEM) of oocytes developed to		
	replicates	examined	≥2 cell	≥4- to 8-cell	blastocyst
–	6	114	77 (67.3 ± 5.2)[a)]	60 (52.5 ± 6.3)[a)]	38 (33.3 ± 3.2)[a)]
+	6	111	91 (82.0 ± 2.3)[b)]	78 (70.3 ± 3.6)[b)]	50 (45.1 ± 2.0)[b)]

* Bovine oocytes were matured with or without VEGF, then fertilized and cultured without VEGF.
+: With 5 *n*g/m*l* VEGF; –: Without VEGF. a, b) Values in the same column with different superscripts differ significantly ($P<0.05$).

control groups. Therefore, the improved development rate from the oocyte to the blastocyst was observed in more of the 4- to 8-cell stage embryos obtained from the group supplemented with VEGF during maturation. These results indicate that VEGF added to the culture medium during IVM enhances the developmental competence of mammalian oocytes.

The first polar body formation and the fertilization rates of oocytes matured *in vitro* are generally lower than those of oocytes matured *in vivo* [23]. This difference is attributed to incomplete cytoplasmic maturation *in vitro* [9]. In the present study, the maturation rate was comparatively higher when bovine COCs were cultured with VEGF. This result suggests that VEGF promotes nuclear maturation. Furthermore, since the cumulus mass expanded well in the group with VEGF, the VEGF-induced nuclear maturation might be mediated by cumulus cells similar to the effect of other growth factors, such as EGF [16]. However, as observed in a previous study [14], EGF does not promote the proportion of normally fertilized oocytes (oocytes with one sperm tail, one male and one female pronuclei) after co-incubation. In Exp. 2 of the present study, the penetration and normal fertilization rates of bovine oocytes were significantly higher ($P<0.05$) in VEGF groups than those in the control when the oocytes were treated with 5 *n*g/m*l* VEGF during maturation or during maturation and fertilization. Furthermore, the subsequent development of bovine oocytes matured with VEGF was improved in Exp. 3. These results suggest that VEGF induces cytoplasmic maturation as well as nuclear maturation in bovine oocytes.

Hainaut *et al.* [14] postulated that the effect of IGF-I on maturation is initiated upon activation of the membrane receptor for this growth factor and requires tyrosine dephosphorylation of p34, the kinase component of Maturation Promoting Factor. It was not clear from this experiment whether VEGF had an effect on the oocyte directly or indirectly via cumulus cells. The mechanism of the effect of VEGF on the maturation of bovine oocytes seems complex compared with IGF-I and further study will be necessary for its elucidation.

In conclusion, VEGF stimulated the maturation of bovine oocytes *in vitro*, resulting in promotion of the normal fertilization rate and the subsequent development of fertilized oocytes. We therefore suggest that VEGF is an important factor not only for nuclear maturation but also for cytoplasmic maturation of bovine follicular oocytes.

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