Vascular Endothelial Growth Factor (VEGF) Promotes the Early Development of Bovine Embryo in the Presence of Cumulus Cells

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ABSTRACT. Three experiments were conducted to determine the effect of Vascular Endothelial Growth Factor (VEGF) on bovine embryonic development *in vitro*. Human recombinant VEGF₁₆₅ was employed at 5 ng/ml in modified synthetic oviduct fluid. In Exp. 1, bovine cumulus oocyte complexes were matured with or without VEGF for 22 hr, inseminated without VEGF for 6 hr, then cultured with or without VEGF for 42 hr. The cleavage rate and the development rate to 4- to 8-cell were higher ($P < 0.05$) in groups with VEGF during *in vitro* maturation (IVM, 71.4% and 59.6%), *in vitro* culture (IVC, 70.3% and 62.3%), and both IVM and IVC (75.9% and 67.8%) than in the group cultured thoroughly without VEGF (49.9% and 38.4%, respectively). In Exp. 2, 4- to 8-cell embryos produced *in vitro* without VEGF were removed from cumulus cells at 48 hr post-insemination (Pi) and cultured with or without VEGF for 144 hr. The development rates to blastocyst at 96 hr (D6), 120 hr (D7) and 144 hr (D8) were similar ($P > 0.05$) in both groups. In Exp. 3, cumulus cells were removed from presumptive embryos produced by IVM and IVF without VEGF at 10 hr Pi. Denuded embryos were cultured with or without VEGF for 38 hr or 182 hr. The cleavage rate and the development rates to 4- to 8-cell at 48 hr Pi and to blastocyst on D6, D7 and D8 were similar ($P > 0.05$) in all groups. These results suggest that VEGF has a beneficial effect on the initial development of bovine embryo through surrounding cumulus cells.

KEY WORDS: bovine embryo, cumulus cell, *in vitro* development, VEGF.

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Bovine oocyte matures at the time of pre-ovulatory gonadotropin surge *in vivo* [4], but maturation of oocyte depends on interaction of many factors. Although gonadotropin is the primary regulator of nuclear maturation in mammalian oocyte *in vivo* [19], many reports imply that gonadotropin is only one in a complex sequence of factors that appear to regulate oocyte maturation and embryonic development. The oviduct provides optimal microenvironments for fertilization of gametes and the development of zygote. The oviductal fluid includes various proteins and growth factors [11], which influence sperm capacitation, final oocyte maturation, fertilization and early embryonic development. However, the composition and the amount of fluid produced by bovine oviduct change depending on the stage of estrous cycle [12]. Expression of growth promoting factors secreted by the oviduct, such as Epidermal Growth Factor (EGF), Fibroblast Growth Factor (FGF), Transforming Growth Factor (TGF) $\beta 1$ and Vascular Endothelial Growth Factor (VEGF) differs in animal species [10, 13].

VEGF is known as a potent mitogen for vascular endothelial cells [6, 18] and as a stimulator of vascular permeability [3]. Expression of VEGF is mainly correlated with the vascularization of tissues [21]. In the female reproductive system, it is essential for the development of follicle and corpus luteum as well as the establishment of the placenta [7]. In cattle, VEGF transcripts continuously increase in granulosa cells accompanied with follicular development

[5] and it shows a beneficial effect on maturation of oocyte *in vitro* [20]. VEGF is also involved in creating optimal local environments for fertilization and the development of bovine embryo by modulating permeability within oviduct [9, 10]. However, the roles of VEGF on bovine oocyte maturation and early embryonic development is elucidated very little.

In our previous study, it was demonstrated that recombinant human VEGF₁₆₅ improved maturation, normal fertilization and subsequent development of bovine oocytes cultured with cumulus cells [20]. However, it has not been determined whether VEGF influences directly to the oocyte or indirectly via cumulus cells. Therefore, the objectives of the present study were to examine whether VEGF was involved in the early development of bovine embryo without oviduct and to determine whether the effect of VEGF was directly on the embryo or indirectly through surrounding cumulus cells.

MATERIALS AND METHODS

Reagents: Human recombinant VEGF₁₆₅ derived from Sf 21 insect cells was obtained from R&D systems (Minneapolis, MN, U.S.A.) and utilized at 5 ng/ml. For *in vitro* culture of bovine oocyte and embryo, synthetic oviduct fluid (SOF) [24] 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 -non-essential amino acid (MEM-NEAA, Sigma M-7145), 5 mM taurine (Sigma T-7146), 0.5 mM pyruvic acid (Sigma P-

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4562), 100 UI/ml penicillin (Sigma Pen-K) and 100 µg/ml streptomycin (Meiji Seika, Tokyo, Japan). For *in vitro* fertilization, BO medium [1] 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 hr. Follicular contents were aspirated from small antral follicles (2–5 mm) using an 20-gauge needle attached to a 10 ml disposable syringe, then allowed to settle in a Petri dish and the supernatant was discarded. Only cumulus oocyte complexes (COCs) with multilayered compact cumulus cells were selected for maturation *in vitro*. The COCs were washed 3 times with m-SOF containing 10% Fetal Bovine Serum (FBS, ICN Biomedical, Inc, 29–167–54, Aurora, OH, U.S.A.), 1.5 mM glucose (Dextrose anhydrous, Wako Pure Chemical Industries, Osaka, Japan), 2 µg/ml Follicle Stimulating Hormone (FSH, Antrin, Denka Pharmaceutical, Kawasaki, Japan), 2 µg/ml 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% CO₂ in air.

In vitro fertilization (IVF): Matured COCs 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 AB, Piscataway, NJ) gradient BO medium and centrifuged 15 min at 800 × g, 37°C. The sperms were diluted at a final concentration of 5 × 10⁶ sperms/ml. Gametes were incubated together 6 hr in the BO medium under paraffin oil at 39°C in an atmosphere of 5% CO₂ in air.

In vitro culture (IVC): The presumptive embryos were washed 3 to 4 times with m-SOF supplemented with 1% FBS at 6 hr post-insemination (Pi), then placed each 20 ± 2 in a 100 µl droplet of the same medium under paraffin oil and cultured at 39°C in an atmosphere of 5% CO₂, 5% O₂, 90% N₂ until 48 hr Pi. Then, cumulus cells surrounding embryos were removed gently by a suitable glass pipette. The denuded embryos were washed 3 to 4 times with m-SOF supplemented with 5% FBS, then transferred each 20 ± 2 to a 30 µl droplet of the same medium under paraffin oil and cultured under the same condition for 144 hr (until D8).

Exp. 1. Effect of VEGF on the initial development of bovine embryo: Bovine COCs were matured with or without VEGF, inseminated without VEGF, then cultured with or without VEGF for 42 hr. The effect of VEGF on the initial development of bovine embryo was assessed by the cleavage rate (CR, No. of ≥2-cell embryos/No. of oocytes), the initial development rate 1 (IDR1, No. of ≥4- to 8-cell embryos/No. of oocytes) and the initial development rate 2 (IDR2, No. of ≥4- to 8-cell embryos/No. of ≥2-cell embryos) at 48 hr Pi.

Exp. 2. Effect of VEGF on the further development of bovine embryo: Bovine COCs were matured, inseminated

and cultured without VEGF. Only 4- to 8-cell stage embryos were selected at 48 hr Pi, removed from surrounding cumulus cells and cultured in 5% FBS-m-SOF supplemented with or without VEGF for 144 hr. The effect of VEGF on the further development of bovine embryo was assessed by the development rate to the blastocyst stage at 144 hr (D6), 168 hr (D7) and 192 hr (D8) Pi.

Exp. 3. Effect of VEGF on the development of bovine embryo thoroughly without cumulus cells: The cumulus cells were removed from presumptive bovine embryos produced by IVM and IVF without VEGF at 10 hr Pi using 0.1% hyaluronidase (Sigma H-3506). The denuded embryos were cultured in 1% FBS-m-SOF supplemented with or without VEGF for 38 hr, then cultured in 5% FBS-m-SOF with or without VEGF for 144 hr. The effect of VEGF on the development of naked bovine embryo was evaluated with CR, IDR1 and IDR2 at 48 hr Pi and the blastocyst rates on D6, D7 and D8.

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

RESULTS

In Exp. 1, a total of 690 oocytes were examined in 9 replicates. As shown in Table 1, CR and IDR1 were higher ($P < 0.05$) in groups with VEGF during IVM (71.4% and 59.6%), IVC (70.3% and 62.3%), and both IVM and IVC (75.9% and 67.8%) than those in the group cultured thoroughly without VEGF (49.9% and 38.4%, respectively). The highest CR (75.9%), IDR1 (67.8%) and IDR2 (90.0%) were obtained from the group that VEGF was added to culture media both during IVM and IVC. Two way ANOVA showed that additions of VEGF to culture medium as well as to maturation medium significantly increased CR ($P < 0.05$ and $P < 0.01$, respectively) and IDR1 ($P < 0.001$ and $P < 0.005$, respectively), whereas no significant interaction was detected between the effects of VEGF during maturation and culture as indicated in Table 2 ($P > 0.05$). Furthermore, supplementation of m-SOF with VEGF significantly ($P < 0.01$) raised IDR2 during IVC but not during IVM ($P = 0.36$, Table 2).

As shown in Table 3, 241 of 4- to 8-cell bovine embryos that obtained from IVM, IVF and IVC until 48 hr Pi performed without VEGF were cultured with or without VEGF for 144 hr in 6 replicates in Exp. 2. Development rates to blastocyst were similar ($P > 0.05$) in both groups on D6, D7 and D8, respectively.

In Exp. 3, CR, IDR1 and IDR2 at 48 hr Pi and development rates to blastocyst on D6, D7 and D8 were examined in 6 replicates with cumulus-removed embryos. Neither CR, IDR1, IDR2 nor development rates to blastocyst on any day were different among groups with or without VEGF during IVC until 48 hr Pi or thoroughly IVC until D8 as shown in

Table 1. The effect of VEGF on the initial development of IVM/IVF bovine embryo

Treatment MFC	No. of oocytes examined (n)	No. of embryos		CR	IDR1	IDR2
		≥ 2 cell	≥ 4- to 8-cell			
---	174 (9)	87	67	49.9 ± 3.9	38.4 ± 2.4	77.1 ± 3.9
--+	175 (9)	123	109	70.3 ± 5.8	62.3 ± 5.3	88.9 ± 2.9
+--	170 (9)	121	101	71.4 ± 4.7	59.6 ± 5.2	83.5 ± 2.2
+++	171 (9)	130	116	75.9 ± 4.8	67.8 ± 3.8	90.0 ± 2.2

M: IVM; F: IVF; C: IVC until 48 hr Pi.

n: Number of replicates.

CR: Cleavage rate; IDR1: Initial development rate 1 (Number of embryos ≥ 4- to 8-cell / Number of oocytes); IDR2: Initial development rate 2 (Number of embryos ≥ 4- to 8-cell / Number of embryos ≥ 2 cell)

+: supplemented with 5 ng/ml VEGF; -: without VEGF.

Table 2. Significance (P value) in statistical analyses on Table 1 by two-way ANOVA

	CR	IDR1	IDR2
Maturation ^{a)}	0.0090	0.0043	0.36
Culture ^{b)}	0.015	0.0008	0.0041
Interaction	0.11	0.078	0.59

a) Effect of VEGF added to m-SOF during IVM.

b) Effect of VEGF added to m-SOF during IVC until 48 hr Pi.

Table 4 (P>0.05).

DISCUSSION

Oocyte maturation and embryonic development occur in response to an ever-changing milieu of gonadotropins,

growth factors, steroids, factors secreted by the oocyte or embryo itself and other unknown molecules [2]. Furthermore, there are synergistic effects of factors on those processes. Some studies on synergistic effects of growth factors have been attempted in bovine embryos, such as EGF and TGF β 1 [15], FGF and TGF β 1 [16], Insulin-like Growth Factor-I and Platelet Derived Growth Factor [16], and EGF and FGF [17]. Gabler *et al.* [9] suggested that VEGF might be involved in fertilization and/or early embryonic development by modulating permeability within bovine oviduct. However, far from synergistic effects with other factors, very little is known to date about the effects of VEGF on oocyte maturation and early embryonic development in any species.

The results of the present study indicated that 5 ng/ml of recombinant human VEGF₁₆₅ added to maturation medium significantly (P<0.01) increased the cleavage rate of bovine

Table 3. The effect of VEGF on the development of 4 to 8 cell bovine embryo produced *in vitro*

Treatment	No. of ≥4 to 8-cell embryos examined (n)	No. (%±SE) of blastocysts		
		144 hr (D6)	168 hr (D7)	192 hr (D8)
-	121 (6)	38 (31.6 ± 4.7) ^{a)}	58 (47.8 ± 5.8) ^{a)}	69 (57.0 ± 7.0) ^{a)}
+	120 (6)	46 (38.9 ± 5.8) ^{a)}	57 (47.9 ± 5.3) ^{a)}	62 (52.2 ± 6.3) ^{a)}

n: Number of replicates; hr: hours Pi.

+: cultured with VEGF following the removal of cumulus cells at 48 hr Pi; -: without VEGF.

a) No significant difference was detected between values in the same column (P>0.05).

Table 4. The effect of VEGF on the early development of bovine embryos without cumulus cells*

Treatment		No. of oocytes examined (n)	Development to		Development to blastocyst at		
C ₁	C ₂		≥2 cells	≥4- to 8-cells	144 hr (D6)	168 hr (D7)	192 hr (D8)
-	-	116 (6)	90 (77.4 ± 5.6) ^{a)}	76 (65.3 ± 5.4) ^{a)}	37 (32.0 ± 5.1) ^{a)}	51 (43.8 ± 7.5) ^{a)}	61 (52.3 ± 6.5) ^{a)}
+	-	117 (6)	88 (75.2 ± 5.2) ^{a)}	74 (63.0 ± 7.5) ^{a)}	29 (24.9 ± 7.2) ^{a)}	44 (37.5 ± 7.2) ^{a)}	49 (41.7 ± 7.0) ^{a)}
+	+	115 (6)	86 (74.8 ± 6.9) ^{a)}	76 (66.1 ± 6.7) ^{a)}	26 (22.5 ± 2.8) ^{a)}	38 (33.1 ± 3.5) ^{a)}	43 (37.4 ± 4.4) ^{a)}

* Bovine oocytes were matured and fertilized *in vitro* without VEGF and surrounding cumulus cells were removed from presumptive embryos at 10 hr Pi.

n: Number of replicates; hr: hours Pi.

C₁: IVC₁ (*in vitro* culture until 48 hr Pi following the removal of cumulus cells); C₂: IVC₂ (*in vitro* culture after 48 hr Pi).

+: cultured in m-SOF supplemented with 5 ng/ml VEGF; -: without VEGF.

a) No significant difference was detected between values in the same column (P>0.05).

oocytes (CR) and the development rate to the 4- to 8-cell stage (IDR1) when the oocytes were cultured with surrounding cumulus cells as COCs. These are consistent with the results in our previous study [20] and suggest that VEGF has a beneficial effect on maturation of bovine oocyte. Normal fertilization rate of oocytes matured *in vitro* was lower than that *in vivo* [25], which was thought to be caused by incomplete cytoplasmic maturation *in vitro* [8]. VEGF supplementation to maturation medium significantly ($P<0.01$) improved the subsequent development rate of presumptive embryos produced by IVM and IVF in this study (Exp. 1). This result suggests that VEGF induces balanced cytoplasmic and nuclear maturation *in vitro*.

As shown in Tables 1 and 2, VEGF added to culture medium also increased CR ($P<0.05$) and IDR1 ($P<0.001$) if the presumptive embryos were cultured with cumulus cells. In addition, VEGF supplementation during culture significantly ($P<0.01$) elevated the development rate of 2-cell embryos to the 4- to 8-cell stage (IDR2), whereas addition of VEGF to maturation medium did not affect IDR2 ($P=0.36$). These results suggest that VEGF is effective for early embryonic development as well as oocyte maturation in cattle. However, VEGF did not affect the development of 4- to 8-cell bovine embryos to blastocyst without cumulus cells in Exp. 2. Similarly, VEGF supplementation to culture medium had no effect on the development of bovine embryo in IVC following the removal of cumulus cells at 10 hr Pi in Exp. 3. These results imply that the promoting effect of VEGF on the development of bovine embryo comes out through cumulus cells.

In cattle, EGF stimulates germinal vesicle breakdown (GVBD) in COC [19] and the development of embryo to the blastocyst stage [14]. However, it promotes neither GVBD of cumulus-free oocyte [22] nor the development of embryo without feeder cells [23]. Similarly in this study, VEGF showed a beneficial effect on the development of bovine embryo cultured with cumulus cells (Exp. 1), but it had no effect on the development of cumulus-free bovine embryo (Exp. 3). This would appear that the effect of VEGF on the early development of bovine embryo is brought through the same pathway as EGF suggested by Im and Park [14]. The relation between the effects of EGF and VEGF on cumulus cells has not been determined, however.

Oxygen shortage in follicular fluid induces VEGF transcription in bovine granulosa cells [5]. Besides, VEGF receptors have been found on the surface of bovine granulosa cells [5] but not in the oocyte yet. Those findings suggest that VEGF has an autocrine regulation system in a bovine follicle and stimulates cumulus cells to regulate oxygen supply to the oocyte. We hypothesize that VEGF produced by cumulus cells may be insufficient for regulating the local environment around bovine oocyte and embryo *in vitro*, because the number of cells supporting the oocyte and the embryo is fewer *in vitro* than *in vivo*. Consequently, VEGF supplementation to maturation and culture media increased the rate of bovine oocyte maturation and embryonic development *in vitro* in this study.

In conclusion, VEGF enhances bovine early embryonic development as well as oocyte maturation *in vitro*. It is suggested that the beneficial effect of VEGF is brought to the embryo through surrounding cumulus cells.

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