Effect of Vascular Endothelial Growth Factor on Porcine *In Vitro* Maturation

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ABSTRACT

This study was performed to investigate the effect of VEGF on *in vitro* maturation of porcine oocytes. The base medium for IVM, TCM-199 was supplemented with 0.6 mM cysteine, 0.91 mM pyruvate, 10 ng/ml epidermal growth factor, 75 μg/ ml kenamycin, 1 μg/ml insulin and 10% (V/V) porcine follicular fluid (pFF) as a Group A; Group B was consists of Group A plus 5 ng/ml VEGF; Group C was consists of replacement of pFF by 10% PVA and Group D: was consists of Group C plus 5 ng/ml VEGF.

1. The maturation rate was significantly higher ($p<0.05$) in control and VEGF+pFF group than other two groups (76.1± 9.6, 78.9±6.0 vs 60.4±14.2 and 58.3±14.3, respectively).
2. Addition of VEGF without pFF showed a negative effect on oocyte maturation and about 58.26% oocytes were reached to M-Ⅱ stage.
3. In the parthenogenetic development, the cleavage rate was significantly higher ($p<0.05$) in control and VEGF+ pFF group (73.2±1.8 and 64.6±1.1, respectively) than other groups (47.9± 1.8 and 48.3±1.7, respectively).
4. The blastocyst formation rate was significantly higher ($p<0.05$) in VEGF+pFF group (32.6±2.4) compared to control and other groups.
5. There was no significant difference in cell numbers (inner cell mass or trophectoderm) among these groups.

(Key words : VEGF, porcine IVM, parthenogenesis, protein kinase)

INTRODUCTION

Assisted reproductive technology (ART) enables to generate the mature oocytes that have a full developmental potential to term (Schroeder and Eppig, 1984) and *in vitro* maturation (IVM) system is an incredibly a important technology. It is clear that there is a deficiency in IVM technology and needs to improve the media formulation especially for oocyte IVM (Gilchrist and Thompson, 2007). A large numbers of studies have been taken to improve the IVM media with several types of growth factors and cytokines that influences the development of preimplantation embryo *in vitro* and *in vivo* and evaluated their functional roles during preimplantation period (Diaz-Cueto and Gerton, 2001). But the oocytes have the capacity to secret these factors that regulate its development. However, addition of exogenous oocytes secreted factors (OSFs) with traditional IVM media improves the oocyte developmental potential as well (Gilchrist and Thompson, 2007). Also, in *in vivo* condition, the mammalian reproductive system creates optimal microenvironment for the development of putative zygote. The microenvironment is influenced by the some growth factors like Fibroblast Growth Factor (FGF), Epidermal Growth Factor (EGF), Transforming Growth Factor (TGF) β1, Vascular Endothelial Growth Factor (VEGF) and others secreted by oviduct (Gandolfi *et al.*, 1993).

VEGF is a peptide, 45 kDa disulfide-linked homodimeric glucoprotein which is mitogenic for vascular endothelial cell (Neufeld *et al.*, 1999; Ferrara and Davis-Smith, 1997). It is important for cell proliferation in normal and tumor cells and in some cells it is able to promote cell differentiation (Diaz-Cueto and Gerton, 2001). In female reproductive system, it is essential for follicular development and valuable biochemical markers of oocyte maturation (Kawano *et al.*, 2003) and as well as corpus luteum development (Findlay, 1986). The VEGF acts via two tyrosine kinase-family receptors, namely Flt-1 (VEGFR-1) and Flk-1/KDR (VEGFR-2) (Ferrara and Davis-Smyth, 1997; Shibuya, 1995). Inhibition of that receptor in monkey markedly decreases in the follicular angiogenesis (Wulff *et*

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al., 2001). In bovine, it shows the beneficial effect on oocyte maturation *in vitro* (Luo *et al.*, 2002b) and exogenous VEGF application at beginning of 24 hours of IVM significantly improved the cleavage rate and as well as blastocyst development in combination with FSH (Einspanier *et al.*, 2002).

Follicular fluid contains all basic growth factors that are required for *in-vitro* embryo development. (Edwards, 1974). In human follicular fluid, gonadotrophins concentration is higher and that might be enhanced the effects on oocyte maturation and embryo development (Suchanek *et al.*, 1994). Various antioxidant present in the follicular fluid that have a beneficial effect of embryo development and also stimulate the synthesis of cell cycle protein in embryos that support their development *in vitro* (Chi *et al.*, 1998).

Development of porcine embryo derived from *in vitro* is relatively low compared to other species due to high *in vitro* fragmentation rate (Wang *et al.*, 1999). Cytoplasmic fragmentation with developmental arrest is a typical characteristic of cells undergoing apoptosis (Hao *et al.*, 2003). Therefore, IVM is the most critical steps for cytoplasmic and nuclear maturation, and subsequent embryonic development.

The effects of VEGF on endothelial cells have long been known the proliferation, chemotaxis and protection from against apoptosis and act as a cytoprotective agent (Gerber *et al.*, 1998) but role of VEGF on extravascular cell is not well understood. Administration of VEGF and VEGF gene fragment directly to the ovary has been shown to stimulate pre-antral follicular growth and increase the number of preovulatory follicles (Hunter *et al.*, 2004; Shimizu *et al.*, 2003). The recombinant human VEGF improves the maturation, fertilization and also subsequent embryonic development of bovine oocyte (Luo *et al.*, 2002a). But, there is no available information about the effects of VEGF on the porcine oocytes in conjunction with pFF on *in-vitro* embryonic developmental competence. Accordingly, the present study was designed: to evaluate the effect of parthenogenesis on *in vitro* matured porcine oocytes with VEGF along with pFF in the maturation media.

MATERIALS AND METHODS

1. Media and Reagent

Unless otherwise stated, all reagents and chemicals used in this study were purchased form Sigma Aldrich Co. (St. Louis, MO. USA). Tissue culture medium-199 (TCM-199) was purchased from Invitrogen, Grand Island, NY, USA. The NCSU-23 medium supplemented with 0.4 % (wt/vol) Bovine Serum Albumin (BSA) was prepared according to the method of Petters and Wells (1993). The four well culture dishes used in all experiments were purchased form Nunc Inc. (Copenhagen, Denmark).

2. Culture Media

The base medium for IVM, TCM-199 was supplemented with 0.6 mM cysteine, 0.91 mM pyruvate, 10 ng/ml epidermal growth factor, 75 μg/ml kenamycin, 1 μg/ml insulin and 10 % (V/V) porcine follicular fluid (pFF) as a Group-A; Group-B was consists of Group-A plus 5 ng/ml VEGF; Group-C was consists of replacement of pFF by 10 % PVA and Group-D: was consists of Group-C plus 5 ng/ml VEGF.

3. Oocytes Collection and *In-Vitro* Maturation

Ovaries were collected from a local slaughterhouse and washed with physiological saline supplemented with 100 IU/ml penicillin G and 100 mg/ml streptomycin sulphate. The ovaries were maintained at 30 to 35℃ in physiological saline during transport to the laboratory (2 hours after collection). Upon arrival the ovaries were washed thoroughly in pre-warmed saline and ere kept at 37℃ during aspiration. The cumulus oocyte complexes (COCs) were aspirated using an 18-gauge needle attached to a 10 ml disposable syringe (Kim *et al.*, 2004) from superficial transparent follicles 3 to 6 mm in diameter and pooled in to 15 ml conical tubes and allowed at 37℃ or 5 minute (Suzuki *et al.*, 2006) to settle down as sediment. The supernatant was discarded and the precipitate was resuspended with HEPES-buffered Tyrode's medium (TLH) containing 0.05 % (w/v) polyvinyl alcohol (TLH-PVA) (Bavister *et al.*, 1983) and observed under a steriomycroscope at 200 magnifications. Only compact COCs with $\geq$3 uniform layers of compact cumulus cells (Bagg *et al.*, 2004; Tong *et al.*, 2004) and uniform ooplasm were recovered from the collected fluid and washed three times in the oocyte maturation medium (OMM) and transferred 50 COCs to pre-warmed 500 μl of culture medium (TCM-199; Invitrogen Corporation, Carlsbad, CA, USA) which was additionally supplemented with fresh 4 IU/ml pregnant mare serum gonadotropin (PMSG) and 4 IU/ml hCG (Intervet, Boxmeer, Netherland) and was incubated at 39℃ in a humidified atmosphere of 5 % CO_2 in 95 % air. After 22 hours, the COCs were washed with PMSG and hCG-free TCM-199 medium and were cultured with the same medium for another 20 hours. The pFF was collected according to Kim *et al.* (2004) and was

stored at −20℃ until use. At the end of maturation, COCs were denuded with 0.5 mg/ml hyaluronidase for 1 min by gentle pipetting.

4. Assessment of Nuclear Maturation

Completely denuded oocytes were stained with Hoechst-33342 (Bisbenzimide) 10 μg/ml (Tong *et al.*, 2004) for 5 min and observed under epifluorescence microscope (Nickon Corp., Tokyo, Japan) at X100 magnification. Two distinct spots fluorescence would be observed for mature metaphase-Ⅱ (M-Ⅱ) stage of oocytes, one was the nucleus of the first polar body and the second was the chromosomes of the M-Ⅱ spindle of the mature oocytes. Oocytes showing an abnormal chromatin configuration or no chromatin at all after staining were considered as being degenerated and were discarded.

5. Parthenogenetic Activation

After denuding, oocyte maturation was assessed by looking for the presence of a polar body under a stereomicroscope. Only matured oocytes, displaying a polar body with homogenous cytoplasm, were selected for activation. Selective oocytes were washed three times with HEPES-buffered Tyrode's medium (TLH) containing 0.05 % (w/v) polyvinyl alcohol (TLH-PVA) and twice in activation medium, consist of 260 mM manmitol containing 0.01 mM $CaCl_2$ and 0.05 mM $MgCl_2$. Parthenogenetic activation was achieved by placing of the oocytes with polar body in between the 1-mm fusion chamber containing activation medium and applying by 2 consecutive DC pulses of 110 V for 60 μsec by using electro cell fusion model LF 101 (Nepa Gene Co. Ltd., Japan). Activated oocytes were immediately placed in the NCSU-23 medium supplemented with 0.4 % (W/V) BSA and 5 μg/ml cytochalasin-B (C-6762; Sigma) and incubated at 5% O_2, 5% CO_2 and 90% N_2 at 39℃ for 6 h. After that, the oocytes were washed three times with NCSU-23 medium and placed at 25 μl of NCSU-23 medium (Kim *et al.*, 2005) covered with mineral oil and incubated at 5% O_2, 5% CO_2 and 90% N_2 at 39℃ for 168 h. Cleavage and blastocyst formation rate were evaluated under a stereomicroscope at 48 and 168h after activation, respectively. Only embryos with a distinct inner cell mass, trophectoderm and a clear blastocelic cavity without any signs of vacuolation were considered to be blastocyst stage embryos.

6. Assessment the Cells Number in Blastocyst

Blastocyst quality was assessed by differential staining and counting of inner cell mass (ICM) and trophectoderm (TE) cells according to a modified staining procedure of Thouas *et al.* (2001). Briefly, quality blastocysts were selected and treated with permeabilizing solution containing 1% (v/v) Triton X-100 (Sigma-Aldrich) and 100 μg/ml fluorochrome propidium iodide for 10 sec followed by 25 μg/ml bisbenzimide at room temperature for 90 min. Then the oocytes were washed with absolute alcohol at room temperature for 80 min. The stained blastocyst were mounted on a glass slides under a cover slip and examined under an inverted microscope (Nickon Corp., Tokyo, Japan) equipped with epifluorescence. The ICM nuclei labeled with bisbenzimide appeared blue and TE cells nuclei labeled with both bisenzimide and popidium iodide appeared with pink. Any blastocysts without duel stain were excluded from the study.

7. Statistical Analysis

All data are presented as mean±SD and were analyzed by ANOVA followed by Duncan's Multiple Range test using SPSS 12.0. Statistical significance was determined when a P value was less than 0.05.

RESULTS

In the *in vitro* maturation rate, total 689 oocytes were examined with different treatment groups for maturation rate at 7 replicates (Table 1). The maturation rate was significantly higher ($p<0.05$) in control and VEGF+pFF group than other two groups (76.1±9.6, 78.9±6.0 vs 60.4±14.2 and 58.3±14.3, respectively). Although there was no significantly difference between the control and VEGF+pFF group, matured oocytes number of VEGF+pFF group was higher than the control group. Addition of VEGF without pFF showed a negative effect on oocyte maturation

Table 1. Effect of VEGF on nuclear maturation of porcine IVM

Group	Total oocytes examined	Oocytes reached to M-Ⅱ (%)
Control	163	76.11± 9.64[a]
VEGF+pFF	165	78.92± 5.99[a]
PVA	181	60.41±14.17[b]
VEGF+PVA	180	58.26±14.29[b]

[a,b] Values in the same column with different superscripts differ significantly ($P<0.05$).

and about 58.26% oocytes were reached to M-Ⅱ stage.

In the parthenogenetic development (Table 2), the cleavage rate was significantly higher ($p<0.05$) in control and VEGF+ pFF group (73.2±1.8 and 64.6±1.1, respectively) than other groups (47.9±1.8 and 48.3±1.7, respectively). But, the cleavage rate of addition of VEGF without pFF was significantly decreases than VEGF+pFF group. Although the blastocyst formation rate was significantly higher ($p<0.05$) in VEGF+pFF group (32.6±2.4) compared to control and other groups (Table 2), there was no significant difference in blastocyst cell number (inner cell mass or trophectoderm) among these groups (Table 3).

DISCUSSION

Electrical parthenogenetic activation was performed in this study to circumvent the detrimental effect of high rate of polyspermy in porcine *in vitro* fertilization (Wang *et al.*, 1998; Abeydeera and Day, 1997) and also it help to prevent introducing the variable paternal genome during IVF to the first stages of embryo development (Bagg *et al.*, 2004). The cytoplasmic and nuclear maturation is essential for successful *in vitro* embryo development. Mammalian cumulus cells play an important role during oocyte growth and maturation. It knows the oocytes are matured in the ovarian follicle until the ovulation. Maturational ability of the mammalian oocytes is closely related to cumulus cell expansion ability (Yokoo and Sato, 2004) and it help to protect the oocytes from oxidative stress (Tatemoto *et al.*, 2000) resulting in acceleration of the maturation rate. Up to date, all research has been focused only effect of VEGF on *in vivo* oocytes and embryo development elsewhere. Some few researches have been published about *in vitro* effect of VEGF on bovine (Einspanier *et al.*, 2002; Luo *et al.*, 2002a), but the effects of VEGF on porcine oocytes maturation and parthenogenesis is not reported yet. In our experiment, 78.92% oocytes were matured with the VEGF and porcine follicular fluid (pFF). It was significantly increase ($p< 0.05$) than without pFF and/or VEGF in the maturation media. *In vivo* condition, VEGF is secreted from granulosa cells and stores in the follicular fluid that contribute the oocyte maturation (Gruemmer *et al.*, 2005). Without pFF and/or VEGF, the cumulus expansion is reduced (data not shown). In bovine, addition of VEGF with the maturation media helps to extrusion of the first polar body and developmental potential of oocyte after IVF (Einspanier *et al.*, 2002). Therefore, decrease VEGF level in IVM medium might be led to delay oocyte maturation of bovine oocytes (Luo *et al.*, 2002a). Without VEGF and pFF in the IVM media demonstrated the same result in our experiment, the maturation rate was significantly less ($p<0.05$) and also reduced the cumulus cell expansion. This result indicates that the promoting effect of VEGF on *in vitro* development is also required in the porcine cumulus cells expansion.

VEGF is a potent mitogen and acts through its receptors, flt-1 and flk1 (Ferrara and David-Smyth 1997). In bovine, *in vitro* matured oocytes with VEGF showed the significantly increased cleavage and embryo developmental rates (Einspanier *et al.*, 2002). In our experiment, the cleavage and blastocyst formation rates were significantly increased ($p<0.05$) than other two group, but it was similar to control group. This result is agreed with Iwamoto *et al.*, (2005). VEGF have an anti-apoptotic action, and it might be protected from apoptosis by reducing of apoptotic related gene expression during *in vitro* culture (Tran *et al.*, 2002). For maintenance of the normal structure of the cell (eg. alveolar structures), VEGF receptor signal is required (Kasahara *et al.*, 2000). Without VEGF and/or pFF the cleavage and blastocyst formation rate is significantly decreased. As recently, VEGF has an important role in the cyclic growth of the ovarian follicle (Geva and Jaffe, 2000). From

Table 2. Developmental ability of parthenogenetic porcine embryos derived from each IVM treatments

Treatment group	Total oocyte observed	% of cleaved	% of blastocyst
Control	78	73.25±1.79[a]	25.56±5.09[b]
VEGF+pFF	76	64.57±1.09[b]	32.65±2.38[a]
PVA	77	47.93±1.85[c]	23.05±3.14[b]
VEGF+PVA	79	48.31±1.67[c]	21.38±2.39[b]

[a,b] Values in the same column with different superscripts differ significantly ($P<0.05$).

Table 3. Cell numbers of parthenogenetic porcine blastocysts derived from each IVM treatments

Treatment group	Total blastocyst evaluated	Cells/blastocyst	
		ICM	TE
Control	11	10.36±1.5	56.18±19.82
VEGF+pFF	17	11.76±4.62	46.41±23.22
PVA	11	10.36±2.38	47.55±22.43
VEGF+PVA	11	11.55±1.69	48.62±20.19

this result indicate that, other growth factors and protein/kinase in the FF are expected to have important for local influences on developing oocytes as suggested early in an IVM system (Otte *et al.*, 2006). But only in human follicular fluid high density lipoprotein associated sphingosine 1 phosphate was identified though in porcine follicular fluid it is still unidentified. So, pFF helps to markedly expansion of the cumulus cells and, at the same time, exogenous VEGF helps to nuclear maturation (Luo *et al.*, 2002a). Therefore, this results suggested that alone VEGF alone could not affect on IVM, and it needs some proteins in the pFF to active VEGF.

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