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EFFECT OF VASCULAR ENDOTHELIAL GROWTH FACTOR 165A ON PORCINE PRIMORDIAL FOLLICLE DEVELOPMENT *IN VITRO*: A PRELIMINARY STUDY

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ABSTRACT

Folliculogenesis is a complex process of ovarian follicle growth and development, which is yet to be completely understood. Vascular endothelial growth factor (VEGF) is known for its ability to promote angiogenesis. Among different candidates, VEGF165a is recognized as one of the major factors that determines the degree of vascularisation of the target tissue. This brings up the importance of studying the role of VEGF165a in ovarian follicle activation with special emphasis to ovarian cortex. The objective of current study was to determine the effect of VEGF165a on porcine primordial follicle viability *in vitro*. This preliminary data was obtained from short-term (72 hours) *in vitro* culture of porcine ovarian cortical strips under 5% CO_2 and 95% O_2 under the humidified atmospheric conditions. Cortical strips were treated with 0.1ng/ml VEGF 165a, 1.0ng/ml VEGF 165a, 10.0ng/ml VEGF165a. Parallel to the treatments, a negative control(Tissues were fixed on 10% neutral buffered formalin) and a positive control were conducted.In this study, all the observations suggested that the lower concentrations of VEGF165a has increased the follicle viability among all treated groups (0.1ng/ml,88.02%; 1ng/ml, 67.68%; 10ng/ml, 25.21%) while higher concentrations implicit higher follicle degeneration (74.79%). In conclusion, the lowest VEGF165a concentration has increased the follicle viability while the highest concentrations implicit increased follicle degeneration in this study.

Key words: Follicle activation, Porcine, Primordial follicle,Viability, VEGF165a

INTRODUCTION

Folliculogenesis is defined as the process of ovarian follicle growth and oocyte development which is a complex process, where the process of activation of cohort follicles are poorly understood (Magamage, 2011a, b). Vascular endothelial growth factor (VEGF) is known for its ability to promote the growth of vascular bed. Formation of new blood vessels or the angiogenesis is a fundamental requirement for the growth and development of any multicellular living organism. Since the primordial follicle is a cumulative cellular structure, further growth and development of this cell structure is largely depended on the accessibility and the availability of the nourishment through the vascular system. Implications of various growth and regulatory factors on primordial follicle activation under intense investigation but implications of vascular endothelial growth factor 165a (VEGF165a) on primordial follicle activation are inconclusive.

Translation biology has recently become very important recently for its applicability for both human health and animal production context. Pig model has been identified as the most physiologically-mimicking mammalian species compared to human model and it was under vigorous investigation as major human organ donor for the future (Reardon, 2015). Further proangiogenic and antiangiogenic nature of VEGF has a significant role in success on xenotransplantation in which the technique purely successful if devoid of tissue rejection

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(Henry *et al*, 2015). Therefore, studying the effect of VEGF on porcine primordial follicle development not only important for reproductive biotechnology but also translation medicine. The objective of the current study was to determine the effect of vascular endothelial growth factor 165a on porcine primordial follicle viability *in vitro.*

MATERIALS AND METHODS

Collection of ovarian cortical stripes

Ovaries were collected from the local slaughterhouse located in Avissawella. Prepubertal ovaries were selected based on slaughter age (5 -6 months old) and surface morphology of the ovaries (flat smooth surface) and transported under the thermos flask within minimum period of time (2.5-3hours) to the laboratory. The ovaries were washed once in 0.2% cetyltrimethylammonium bromide (Sigma, MO, USA) and Dulbecco's phosphate-buffered saline (PBS) supplemented with 0.1% polyvinyl alcohol (PVA; Sigma, MO, USA) 3 times. Cortical strips from randomly selected ovaries, with a thickness of 0.5 mm (approximately) were dissected and selected under microscope (Carl Zeiss Stemi 2000™, Germany). Cortical strips containing mainly primordial follicles were selected while strips containing primary and secondary follicles were excluded from culture. All the cortical sections were divided into two (2) parts and one portion was immediately fixed (negative control at day 0) in 10% Neutral Buffered Formalin (NBF;Sigma, MO, USA) while other part was subjected to further processing for culture.

In vitro culture

Primordial follicle containing cortical strips were again grouped into four (4) according to four different dose regimes; VEGF 165a 0.0 ng/ml (Positive Control), 0.1 ng/ml, 1.0 ng/ml and 10.0 ng/ml. These tissues were cultured in 24 well polystyrene tissue culture plates after placing 6-8 tissue sections top of the cell culture inserts. Basic culture medium α-MEM was supplemented with 5% bovine serum albumin (BSA), 1% Insulin-Transferrin-Selenium (ITS) and 0.08% Kanamycin. Ovarian cortical strips were cultured for 3 days under humidified atmosphere of 5% CO_2 and 95% air at 38.5°C. Followed the model described in Magamage *et al*. 2011.

Assessment of follicular development

The ovarian cortical strips (before and after culture) were fixed in 3% paraformaldehyde in PBS, then dehydrated, embedded in paraffin, serially sectioned by 5 µm, and stained with hematoxylin and eosin. Then the number of follicles in different stages were counted under the microscope (Carl Zeiss Stemi™, Germany) and recorded.

The follicles were classified into five categories according to the number and morphology of granulosa cell layers: Single layer of flattened granulosa cells surrounding the oocyte: Primordial follicles, Single layer of cells containinga mixture of flat and cuboidal granulosa cells: Intermediate follicles, Single layer of cuboidal granulosa cells surrounding the oocyte: Primary follicles, Follicles with two or more granulosa cell layers surrounding the oocyte considered as Secondary follicles. Follicles having oocytes with pale cytoplasm, shrunken or extensive cytoplasmic vacuolations were considered as degenerated follicles.

Data analysis

Data were analyzed by one-way ANOVA, using SAS 9.0 software following the 95% significance level and there weren't any significance differences among treatments.

RESULTS AND DISCUSSION

Out of three different dose regimes, 0.1ng/ml showed the highest numbers of viable follicles at the end culture period compared to 1ng/ml and 10ng/ml (Figure 1).

More follicles were able to transit to advance stages in lower VEGF 165a concentrations compared to higher concentrations. Dose regime of 10.0ng/ml VEGF165a treatment

demonstrated an accelerated follicle degeneration (Table 1).

There are many factors regulate follicle viability in porcine ovary *in vitro*. Magamage *et al*

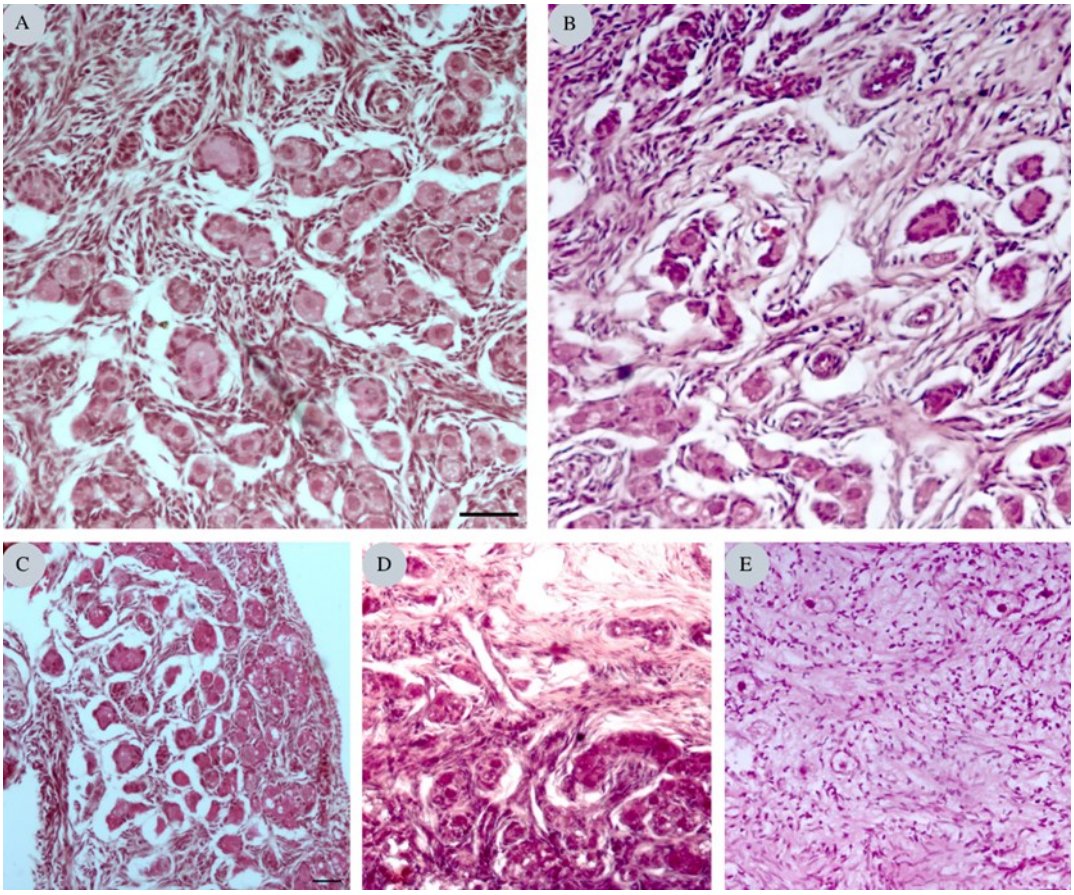

Figure 1: Microscopic images of with or without VEGF 165a treated porcine ovarian cortical tissues. (A: Before Culture, B: Control, C: 0.1ng/ml VEGF 165a, D: 1.0ng/ml VEGF 165a, E: 10.0ng/ml VEGF 165a, Scale bar represents 40µm.)

Table 1: Effect of VEGF165a treatment on development and viability of porcine primordial follicles.

Treatment	Days of culture	No of Tissues Examined	Ave. No of Follicles Per Tissue	Number of follicles*					
				Primordial	Int:	Primary	Secondary	Degenerated	Cell Viability %
Control (Day 0)	0	6	40.3	(25.5 ± 0.16)	(5.7 ± 0.15)	(6.8 ± 0.13)	(0.3 ± 0.15)	(2 ± 0)	95.03722
Control 0.0 ng/ml VEGFA	3	6	31.2	(18.55 ± 0.17)	(5.33 ± 0.16)	(3.55 ± 0.17)	(0.11 ± 0.11)	(3.66 ± 0.16)	88.26923
0.1ng/ml VEGFA	3	6	30.9	(16.9 ± 0.27)	(3.4 ± 0.16)	(5.2 ± 0.13)	(1.7 ± 0.15)	(3.7 ± 0.26)	88.02589
1.0ng/ml VEGFA	3	6	30.2	(16.72 ± 0.19)	(2.18 ± 0.26)	(1.27 ± 0.14)	(0.27 ± 0.14)	(9.72 ± 0.23)	67.68212
10.0ng/ml VEGFA	3	6	21.1	(4.33 ± 0.16)	(0.66 ± 0.23)	(0.22 ± 0.14)	(0.11 ± 0.11)	(15.77 ± 0.27)	25.21327

(2011) reported that stem cell factor also known as kit ligand mainly regulate the follicle viability in porcine tissues. Higher VEGF165a concentrations may have deleterious effect on intrinsic kit ligand activity which is a leading cause for excessive tissue degeneration under the current study. Current finding in the lowest concentrations are in agreement with previous findings in cows (Ayoub *et al*. 2016). Most probable other explanation for excessive follicle degeneration under higher concentration of VEGF165a may be due to the change of intrinsic tissue balance between proangiogenic and antiangiogenic VEGF165a: VEGF165b ratio. VEGF165b is proposed to be rich with antiagiogenic properties. It is important to have further in-depth studies with incorporation of stem cell factor and VEGF165b in future studies.

CONCLUSION

The lowest concentrations of VEGF165a has increased the follicle viability while higher concentrations increased the follicle degeneration. Further studies are necessary to elucidate the underline molecular mechanism of primordial follicle activation and follicle viability *in vitro* under this proposed model.

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