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The effect of progesterone treatment after ovarian induction on endometrial VEGF gene expression and its receptors in mice at pre-implantation time

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ABSTRACT

Objective(s): Progestrone is a prequisite for pre-implantation angiogenesis and induce decidual angiogenesis. It is unknown the effect of progestrone administration on the endometrium of hyperstimulated mice at pre-implantation time.

Material and Methods: Adult female NMRI mice were divided in three groups [control group, ovarian stimulated group and progestrone treated mice after ovarian stimulation]. Uterine horn samples removed at pre-implantation time in each group. Motic image Plus 2 software was used to assess the quantitative vascular parameters of endometrium. Gene expression was determined for vascular endothelial growth factor (VEGF), FMS-like tyrosine kinase (FLT) and Kinase insert domain protein receptor (FLK) genes using the real time PCR method. Data analysis was done with LinReg PCR and Rest-RG software.

Results: Comparison between progestrone treated mice after ovarian stimulation with control group showed that increase in rate of VEGF gene expression [0.775] and decrease in rate of FLK [6.072] and FLT [1.711] gene expression. Analysis of the data on quantitative vascular parameters were indicated remarkable increase in quantitative vascular parameters of progestrone treated mice compare to control group.

Conclusion: Biological effect of progestrone on the vascular changes after ovarian stimulation resulted in an increase in VEGF receptors experession, it seems that induced angiogenesis by progesterone could result in better condition for implantation.

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Introduction

The angiogenic effect of vascular endothelial growth factor [VEGF], mediated by two receptors, FMS-like tyrosine kinase (FLT) and kinase insert domain protein receptor (FLK) receptor that play important role for implantation in decidual angiogenesis and uterine vascular permiability (1). Hormonally controlled angiogenesis is fundamental for endometrial development and the differentiation which is prerequisite for implantation (2). Endometrial angiogenesis affected by estrogen [E2] for the growth of vasculature at proliferative phase while affected by progestrone [P4] for growth of coiled arteries at secretory phase (3). Uterine epithelial cells and endometrial stromal cells are known as the source of VEGF that directly affect angiogenesis by inducing endothelial progenitor cells (3, 4). The main source of the stromal VEGF are decidual cells that followed by pre-inflammatory condition and presence of macrophage and uterine natural killer [uNK] cells as secondry sources of VEGF (5-7). VEGF is a survival factor for endothelial cells which prevent program cell death induced by serum deprivation (8). It seems that usage of gonadotropin releasing hormone analogs to produce multifactorial receptivity (9). Supraphysiological level of E2 is the reason for insufficiency in patients with high gonadotrophin response (10). It was declared that VEGF expression and its receptor, Flk, were increased in the ovaries of the hyperstimulated rats (11, 12). Ovarian stimulation indicated inhibitory effect on endometrial angiogenic index during implantation window but increased rate of VEGF expression may be due to regulatory role of the ovarian hormones on vascular permeability for implantation (3, 12). GnRH agonist administration reduced VEGF and receptors in the hyperstimulated rats. These suppressions may be mainly due to a direct effect of GnRH on the ovaries (13). Ovarian hormones like E2 and P4 are basically involved in the confirmation and maintenance of pregnancy (14). It is obvious that P4 is a pre-requisite for peri-implantation angiogenesis and induce

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decidual angiogenesis (15, 16). Uterine changes mediated by progestrone is necessary for embryo implantation at early pregnancy lead to proliferation of endometrial endothelial cells and angiogenesis (5). Estrogen enumerate as inducer for progestrone receptor expression in the endometrium (17). P4 is able to induce decidualization and FLK expression in mice. Also, increased VEGF expression were observed in the epithelium of the endometrial cells which are P4 positive. It was followed by an increase in the vascularity and blood flow (18-19). E2 up regulates FLK expression in the endothelial cells of endometrium mainly through the regulation of VEGF by a paracrine mechanism (2). Whereas it is unknown the role of P4 administration in the ovarian stimulated mice on the expression of angiogenic gene and it's receptors in the ovarian stimulated mice at pre-implantation time. We will assess the effect of P4 administration on the expression of VEGF and it's receptors, FLT and FLK in the ovarian stimulated mice at pre- implantation period.

Materials and Methods

Animals: Adult female (NMRI, 6–8 weeks old) mice were cared and housed for 2 weeks in the animal house for better adaptation. Mice were divided accidentally into three groups, Control group, ovarian stimulated group and progestrone treated mice. Pseudopregnancy were induced in the control group. Mice in the ovarian stimulated group received human monopausal gonadotropin (HMG) and human chorionic gonadotropin HCG (7 IU, IP), then pseudopregnancy induction was done. Mice in the progestrone treated group received progestrone (1 mg/mouse) (20) daily for 3 days following the induction of ovarian stimulation and pseudopregnancy. Finally mice were sacrified by cervical dislocation in the each group at pre-implantation time.

Histological preparation

The tissue samples were obtained from the uterine horns. Paraffin sections were prepared by serial section method. For assessment of quantitative vascular parameters, the sections were stained by hematoxylin and eosin (H&E). Motic image software was used to assess the vascular area. For vascular density counting, at least 5 sections in each sample were selected randomly and five microscopic field in the each section were analyzed with magnification x400.

RNA isolation and cDNA synthesis

Extraction of RNA was performed by RNeasy Mini Kit, according to manufactory company catalog (Qiagen Cat. No. 19306). The purity and concentration of RNA was performed by measuring the absorbance at 260 nm [A_{260}] in a spectrophotometer. cDNA synthesis was performed by the unique QuantiTect Reverse Transcription Kit, according to manufactory company catalog [Qiagen Cat. NO. 205311 Thermo Scientific K 1621 Lot 00124084]. Concentrations of RNA (µg/µl) were between 1.2-1.4 and purity of RNA (Absorbance 260/280 nm) was between 1.8-2. List of mouse primers used in Real time-PCR was shown in Table 1.

Real-time PCR

Real-time PCR was performed by using Rotor-Gene Q with SYBR Green detection for gene expression analysis. Forty reaction amplification cycles was performed. Each reaction cycle consisted of: 15 sec at 95 ˚C and 60 sec at 60 ˚C. Control mixture consisted of PCR mixture without cDNA. Gapdh gene was used as an internal control.

Statistical analysis

The results obtained from the histological study in each group were compared using Mann-Whithny test. Statistical analysis was done using SPSS statistical software and $P<0.05$ were considered statistically significant and also the results obtained from the real-time PCR was assessed through the rest-RG software.

Results

Histological findings

The results of present study showed that significant difference ($P\leq0.05$) in the vascular density between the control (1.83±0.4) and progesterone treated group (5.28±0.42), also significant difference ($P\leq0.05$) was observed between the ovarian stimulated group (2.83±0.47) and progesterone treated group (5.28±0.42) at pre-implantation time. Analysis data on the internal vascular area showed significant difference ($P\leq0.05$) between the control (51.43±4.43) and progesterone treated group (109.22±9.46). Data was shown in Table 2 and Figure 1.

Real-time PCR

VEGF, FLK and FLT gene expression were determined using the method of real-time PCR.

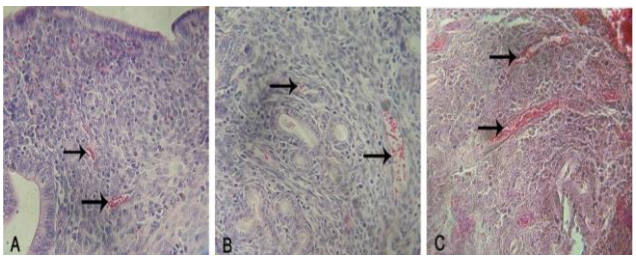

Figure 1. The micrograph of endometrium was shown at pre-implantation time. Micrograph shows vascular density and internal vascular area stained with H&E. control group [A], Ovarian stimulated group [B] and P4 treated group [C]. The black arrows show vascular section. [Magnification X400]

Table 1. Mouse primers used in real time-PCR studies

Gene	Sequence 5'---► 3'	Lenght	Annealing temperature	Gene bank code
Real-Time RT-PCR primers				
Glyceraldehyde-3-phosphate dehydrogenase Gapdh)	F:ATCACTGCCACCCAGAAGAC	20	59.67	NM_001289726.1
	R:AGATCCACGACGGACACATT	20	59.10	
Vascular endothelial growth factor A(VEGF)	F:GGAGACTCTTCGAGGAGCACTT	22	61.46	NM_001110268.1
	R:GGCGATTTAGCAGCAGATATAAGAA	25	59.36	
FMS-like tyrosine kinase 1 (FLT-1)	F:GAGGAGGATGAGGGTGTCTA	20	57.24	NM_010228.3
	R:GTGATCAGCTCCAGGTTTGA	20	57.52	
Kinase insert domain protein receptor [KDR]	F:GGCGGTGGTGACAGTATCTT	20	59.75	NM_010612.2
	R:GAGGCGATGAATGGTGATCT	20	57.46	

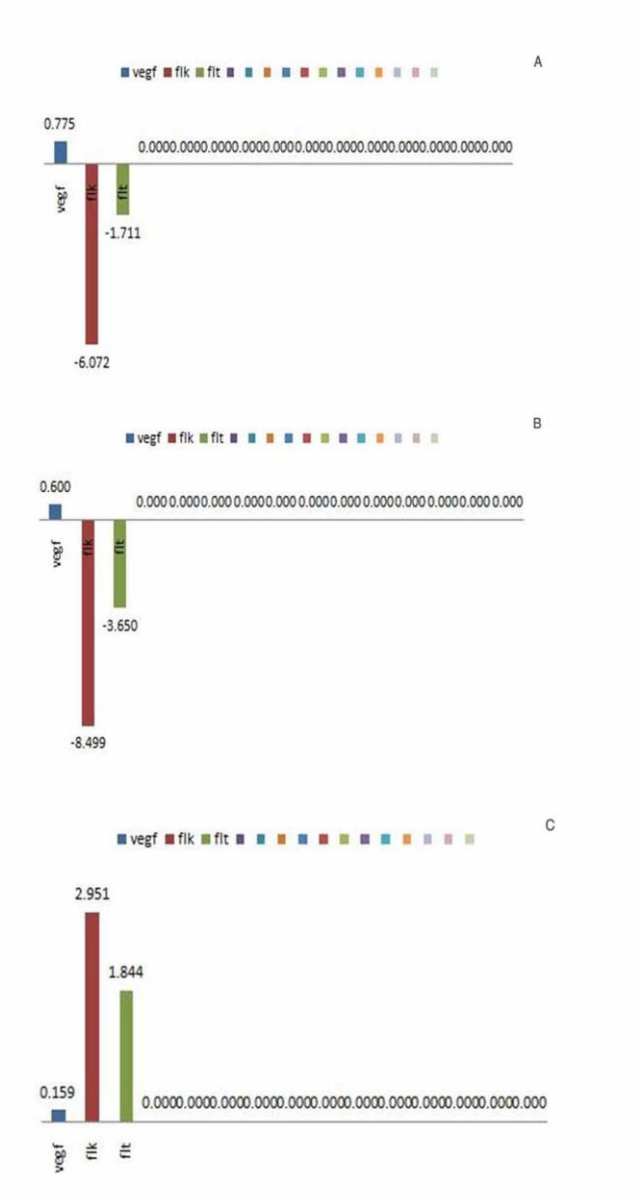

Figure 2. Quantitative expression analysis of the VEGF, FLK and FLT . These graphs were applied with rest-RG software . uterine horn obtained from the control and progesterone treated groups. (A), uterine horn obtained from the control and hyperstimulation groups (B) and uterine horn obtained from the hyperstimulation and progesterone groups (C)

Results were analyzed with LinRegPCR and Rest-RG softwares. Comparison between progesterone treated mice with control group showed increase in rate of VEGF gene expression (0.775) and decrease in rate of FLK (6.072) and FLT (1.711) gene expression (Figure 2 A). Analysis data on comparison between hyperstimulated mice with placebo group showed increase in rate of VEGF gene expression (0.600) and decrease in rate of FLK (8.499) and FLT (3.650) gene expression (Figure 2 B). VEGF gene expression (0.159) was also increased in progesterone treated mice compared to than hyperstimulated group while rate of FLK (2.951) compared to and FLT (1.844) gene expression was decreased in progesterone treated mice compared to than hyperstimulated group (Figure 2 C).

Discussion

Histological findings of present study indicated significant difference in the vascular density of the progesterone treated mice compare to the ovarian stimulation and control group. Beside, intra vascular area assessment was compatible with these results as the progestrone administration resulted in significant difference compare to the ovarian stimulation and the control group. It is inferred that the P4 administration resulted in elevated angiogenesis. In some experimental studies it was evaluated that growth of coiled arteries at the secretory phase affected by progesterone (3, 4). Beside, other researchers reported that microvessel density slightly suppressed after progestin treatment whereas number of stabilized vessels increased (21). These reports that mentioned above are compatible with the results of our study. It was shown that vascular plexus formation start with proliferation and differentiation of stromal fibroblast cells, finally endothelial cells proliferation will happen within the decidua (22). It seems that the progesterone treatment after ovarian stimulation resulted in decidual angiogenesis which our molecular findings confirmed histological studies. Gene expression findings in our study demonstrated that highest

Table 2. Comparison of quantiative vascular parameters was shown in the endometrium of different groups of mice at pre-implantation time

Groups	Control group	Ovarian stimulated group	P4 treated group
Vascular density	*1.83 ±0.4	2.83 ± 0.47**	*5.28± 0.42**
Internal vascular area	51.43±4.43	70.91± 5.27*	*109.22± 9.46

Data are expressed as the means± SD. *P<0.05, significantly different from control group (n= 5 per group) and **P<0.05, significantly different between ovarian stimulated group with p4 treatment group after ovarian stimulated group (n=5 per group)

VEGF gene expression observed in the P4 treatment group. Although quantitative comparison of the FLK and FLT receptors expression are indicative of positive expression of these receptors in the P4 treatment group compared to than ovarian stimulated group but quantitative comparison of these receptors showed negative expression between other groups. Another study implied that ovarian stimulation resulted in enhancement of VEGF and Flk gene expression in the ovaries of rats (11). Also, some researchers concluded that VEGFR1 (Flt-1) and VEGFR2 (Flk) are requisite for the decidual angiogenesis at implantation site. In fact, they reported that blockade of these receptors lead to decrease in the vascular density and disrupt the embryo development and pregnancy (22, 23). The results presented here showed that P4 treated mice lead to an increase in the VEGF gene expression, its receptors and vascular density compared to than ovarian stimulated mice. On the other hand, a decrease in the VEGF receptors was observed in the P4 and and ovarian stimulated mice compare to control group. These findings clarify a decrease in VEGF receptors despite of VEGF gene expression. In fact, biological effects on the vascular changes in the P4 treated mice after ovarian induction resulted in an increase in the VEGF receptors gene expression that is supported by Meduri and colleagues findings (24). They demonestrated that an increase in the VEGF receptors is needed after enhancment of microvascular density and vascular permeability during implantation at secretory phase. In attention to performed studies, we guess that this idea supported by regulatory role of the P4 for VEGF secretion and decidual angiogenesis. Also, another experimental study showed that induced angiogenesis by P4 could enhance emberyo receptivity at this time (18). Recently, one study reported that biological effect of VEGF on the endometrium is attributed to enhancement of VEGF/VEGFR1 ratio that act as multiplier on the angiogenic media (25). Our findings about this ratio in the present study is indicative of the effect of progesterone on the angiogenic media. Recent study exhibited that responsive uterus to ovarian hormones assosiated with an increase in the VEGFR2 (Flk) and angiogenesis, moreover resulted in an increase in the VEGF gene expression (2). However in our findings P4 treatment after ovarian stimulation resulted in a decrease in the VEGF receptors which certainly modified hormonal levels compare to physiological condition. As a consequence, an increase in the VEGF receptors in the present study is indicative of endometrial microvasculature response to P4 treatment after ovarian stimulation compare to ovarian stimulation group. Also, it has been reported that some part of reduced VEGF receptors expression could be due to 2-metoxy estradiol which act as anti-angiogenesis agent. Increased 2-metoxy estradiol associated with endothelial cell apoptosis (26). In addition, some part of increased VEGF receptors in P4 treatment group than ovarian stimulation group could be due to produced proangiogenic factors of NK cells as one of the secondry source of endometrial angiogenesis (27-29). Other studies describe that supraphysiologic levels of estradiol after ovarian stimulation could be accompained by high levels of this metabolite that may be result to decrease in VEGF receptors in endothelial cells of decidua as source of VEGF (3,4). Based on our data, P4 treatment after ovarian stimulation compare to the control group could result in a decrease in the VEGF receptors expression.

Conclusion

It is concluded that induced angiogenesis by progesterone administration after hyperstimulation could reflect the better condition for embryo receptivity through an increase in the quantative vascular parameters and VEGF gene expression in the endometrium at pre-implantation time than hyperstimulated mice which only received HMG and HCG treatment. It seems that P4 administration following HCG and HMG treatment in the IVF patients may improve embryo receptivity at implantation time.

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