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Original Article

Decreased Expression of Endocrine Glands Vascular Endothelial Growth Factor (EG-VEGF) in Rat Endometrial After Stimulation with Recombinants FSH Can be Reduce Implantation Rates

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ABSTRACT

Background: Decreased expression of EG-VEGF in human endometrium after administration of ovarian stimulators has been reported to adversely affect endometrial receptivity and low implantation rates. **Objective:** To determine the effect of recombinant FSH administration on EG-VEGF protein in the endometrium of rats taken in the secretory phase and how it relates to endometrial receptivity. **Design:** A total of 36 female wistar rats with normal estrous cycles were randomly assigned to the natural cycle group (NC) and two stimulation groups (SC) which were injected with recombinant FSH at 12.5 IU and 25 IU intraperitoneally. Uterine necropsy and blood collection were performed on day 1, day 2, and day 3 after hCG administration. A total of 3 female rats from each group were mated with male rats (two males and three females in one cage). A successful marriage is indicated by the presence of a vaginal plug the next day. The level of EG-VEGF protein expression was assessed by immunohistochemical technique and steroid hormone levels were measured by the Elisa technique. **Results:** ANOVA test, that the expression of EG-VEGF in the endometrial glands showed a significant decrease from the normal cycle group to the stimulated cycle group 1 (SC 1) and SC2 (P = 0.00), as well as the expression of EG-VEGF in the endometrial stroma. (P = 000). Steroid hormone levels did not show a significant decrease between the normal cycle group and the stimulated cycle group (P = 0.48 and P = 0.13). **Conclusion:** Decreased EG-VEGF expression in rat endometrium after administration of recombinant FSH is associated with decreased endometrial receptivity which can reduce pregnancy rates.

Key words: rFSH, EG-VEGF, Endometrial receptivity, Secretory phase.

INTRODUCTION

Much progress has been made in assisted reproductive technology such as selection of good quality embryos in the growth medium and production of many mature follicles, however the pregnancy success rate is only around 25-30%.[1,2] The success of implantation and pregnancy depends on cross-talk between the embryo and the endometrium.[3] If embryo selection technology is well developed today, then the non-receptive endometrial environment is a barrier to the success of ART. Transfer of good quality embryos to an unreceptive endometrium is believed to be one of the main reasons behind the failure to establish a pregnancy.[4] The period of receptivity of the endometrium or so-called window of implantation is limited to a short period of time in the menstrual cycle, *i.e.* 6-10 days after the LH surge and lasted for almost 48 hours. During the implantation window period, there are morphological and functional changes that allow the endometrium to adapt to the embryo during the implantation process.

The receptivity of the endometrium has been evaluated through the expression of receptive markers such as biochemical markers, soluble ligands, hormone receptors, cytokines, microRNA or HOX class homeobox genes.[5-10] In addition, endometrial receptivity also influences the occurrence of endometriosis that often found in women of reproductive age.[58] However, it is still limited to the level and pattern of its expression in the implantation window period and the correlation with pregnancy rates has not been explained. It is well known that angiogenic factors contribute to successful implantation and pregnancy outcome. Among these factors, prokineticin is a new target related to ovarian physiology, endometrial receptivity, embryo implantation. In recent years, prokineticin has been shown to play a role in female reproduction and human pregnancy. Its expression in endometrial tissue plays a role in receptivity and increases the ability of the endometrium to accept embryo implantation.[11,12] Prospective studies suggest that prokineticin can facilitate embryo implantation.[13]

Endocrine Gland Vascular Endothelial Growth Factor (EG-VEGF) is a vascular endothelial growth factor that plays an important role in preparing the endometrium for embryo implantation and maintaining the decidua optimally in early pregnancy.[14,15] EG-VEGF has been identified as a novel biomarker of human endometrial receptivity.[16,17] This peptide directly regulates genes involved in implantation and enhances the adhesion of human trophoblast cells to extracellular matrix proteins through the induction of leukemic inhibition factor (LIF). Its expression is detected in steroidogenic glands, such as ovaries, testes, adrenal cortex, and placenta.[18-20] In the endometrium, EG

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VEGF expression is localized to the endometrial luminal epithelium, endothelium and several compartments within the endometrial stroma.[21-23] Its expression pattern in humans is similar to that of progesterone secretion, which starts to increase in the late follicular phase and peaks in the middle of the secretory phase or in the implantation period and decreases at the end of the luteal phase.[24-26] Therefore, this indicator of an unreceptive endometrial environment can be assessed based on EG-VEGF expression in endometrial tissue that is not optimal in the mid-luteal phase.[22,27] Suboptimal expression of receptive markers can interfere with embryo implantation.[28,29] We have conducted a study on changes in EG-VEGF excretion throughout the estrus cycle in a group of female rats stimulated by a single dose of 12.5 IU and 25 IU of recombinant FSH combination (gonal-F) in the diestrus and pergnil phases of 10 IU, 48 hours and compared it with a group of mice in a natural cycle. Objective: To determine the impact of recombinant FSH administration on EG-VEGF protein expression and how it correlates with the number of births.

MATERIAL AND METHODS

Animal experiment

Wistar strain female rats used as experimental animals were approved by the ethics committee of the Faculty of Medicine, University of Indonesia. The handling procedure was in accordance with the recommendations outlined in the guidelines for the care and use of experimental animals. A total of 36 adult female rats and 5 male rats aged about 3.5 months and weighing 150-200 g were acclimatized for 1 week under 12 hours of light; 12-hour dark cycle. Animals were reared in the laboratory unit of the Biomedical Center for Basic Health Technology (PBTDK) at a temperature of 22 ^{0}C, given drinking water and standard pellet food. Every morning, at 09.00, vaginal cytology was performed to determine the estrus cycle of each animal, for 3-5 cycle periods. Female mice without normal estrous cycle periods were excluded from the study and female with regular cycles were used. Female rats with normal estrous cycles were randomly divided into natural cycle groups of two stimulated cycle groups.

The stimulated cycle group was injected with recombinant FSH (Gonal-F) at a dose of 12.5 IU and a dose of 25 IU intraperitoneally (ip) at the beginning of the diestrus phase and continued with injection of hCG (pregnyl), a dose of 10 IU, 48 hours later. Uterine necropsy and blood collection were performed on day 1, day 2, and day 3 after hCG administration. A total of 3 female rats from each group were mated with male rats after administration of hCG, (two males with three females in one cage). A successful marriage is indicated by the presence of a vaginal plug on the following day.

Uterine necropsy and endometrial dating

Prior to surgery, animals were sedated with ketamine 0.1 mL/kg BW. One third of the uterine organs left and right were cut and stored in 10% formalin normal buffer solution (BNF). Then dehydrated in serial alcohol grade, continued in xylene solution and embedded in paraffin. Samples embedded in paraffin were cut to a thickness of 4 m and stained with Hematoxylin-eosin (H-E) staining for histological dating assessment. Histological dating of endometrial tissue was analyzed by observing developments in the luminal epithelial compartment.[30] The assessment criteria are as follows, in the metestrus phase, the luminal epithelium is characterized by high columnar and low in the diestrus phase. During the proestrus and estrus phases thickening and forming a pseudostratified layer, the luminal epithelium reaches its maximum height and cell apoptosis increases.

Immunohistochemistry for EG-VEGF and image analysis

The tissue was embedded in paraffin media and cut into 4 mm thin strips and pasted onto Aurona's premium polysine-coated slides. Next, the process was paraffinized in xylene twice, with each time for 3 minutes and continued with the rehydration process in graded alcohol solution (100%, 96%, 80%, and 70%), for 5 minutes. The slides were stained with hematoclin-eosin stain for endometrial histological dating analysis. The other slides were stained with immunohistochemistry to observe the expression of EG-VEGF protein. After the paraffinization process, the slides were washed with running water, put in methanol liquid with 3% H2O2 content (endogenous blocking) for 10 minutes, then washed again with water. The appearance of antigen on the tissue was carried out according to the One Step protocol of the Neopoly Polymer Detection Kit. At first, the slides that have been washed are stored in a container containing Tris EDTH with a pH of 9. Next, the slides in the container are heated in the Retrieval Generation One BioGear apparatus at a temperature of 98 ^{0}C for 15 minutes. Then, the slides were cooled and washed in phosphate buffered saline (PBS) solution. After that, slides were incubated with polyclonal antibody EG-VEGF dilution 50 times at 40C for 24 hours and washed with PBS solution (clone BZ-0870890F-AP, Bioenzy). Furthermore, the second antibody was added, namely universal polymer HRP and incubated for 30 min. After that, the slide was again washed in PBS solution for 5 minutes, then added DAB and washed with running water. The slides were stained with hematoxylin-eosin (HE) stain and washed with running water. Finally dehydrated in graded alcohol (70%, 80%, and 96%) for 5 minutes each and cleaned by dipping in xylene for 3 minutes. The slide is then covered with entelan. Positive EG-VEGF protein expression was indicated by a brown color that appeared in the cell nucleus when observed under a light microscope.

Each IHC slide was observed using a light microscope with a total magnification of 400X and documented using a computer with Leica Software LAZ EZ and a camera that has been integrated with the Leica Microscope DM750. Photographs were taken at random with a total of five visual fields per one preparation. Ten, the intensity of the brown color is calculated using the plugin program in Figure J, IHC profiler, which will measure the color intensity of an image. The quantification result is converted into an H-Score based on the formula. H-Score = (% positive low x 1) + (% positive x 2) + (% positive high x 3).

Measurement of steroid hormone levels

Hormone levels in rat serum were checked by the elisa method, using the BIOENZT paint reagent. No BZ 08181470-EB, standard curv range 2 ng / ml - 600 ng / ml. The well-bound polyclonal steroid antibody is reacted with progesterone antigen in rat serum and a second antibody labeled with biotin is added. Against the antibody antigen labeled with biotin, streptavidin-HRP was added and incubated. Unbound Streptavidin-HRP will be wasted during washing. The absorbance value of the color that appeared after the addition of the substrate was measured at a wavelength of 450 nm. The sensitivity value for the measurement of the hormone estradiol was 15 pg / mL and the coefficient between examinations was 6%. The sensitivity value for progesterone was 1.046 ng / ml and the coefficient between measurements was <10%. Prior to the examination all serum and reagent samples were stored at minus -20^{0}C.

Gene expression analysis

Gene expression was analysed using quantitative real-time PCR (qPCR). Total RNA was extracted from all samples using Total RNA Mini Kit for Tissue samples (Geneaid, Taipei, Taiwan). Both quality and quantity of the RNA were determined using Nanophotometer implen. cDNA was synthesized from 5 ug of total RNA using Toyobo (Japan) according to manufacturer's protocol. The sequens of specific primers are listed at Table 1. All reactions were performed using 20 ng cDNA, 10 ul of SensiFAST SYBR No-Rox (Bioline, Meridian Bioscience, Ohio, USA) and sets of primers at the optimized concentrations. Final reaction volumes were made up to a total volume of 20 ul with RNAse-

Table 1: Mean EG-VEGF expression in endometrial glands and stroma, levels of estradiol, progesterone and number of births in natural cycle (NC) and stimulated cycles (SC) groups.

Groups	NC	SC1	SC2	P value
H-Score EG-VEGF in gland	288.36	215.85*	184.41*	P < 0.05
H-ScoreEG-VEGF in stroma	272.95	212.15*	196.75*	P < 0.05
mRNA EG-VEGF (ug/ml)	3.76801E-05	2.09039E-07	2.32359E-06	P > 0.05
Estradiol (pg/mL)	46.25	51.51	44.44	P > 0.05
Progesteron (ng/mL)	62.95	82.75	67.38	P > 0.05
Birth	10	10	4*	P < 0.05

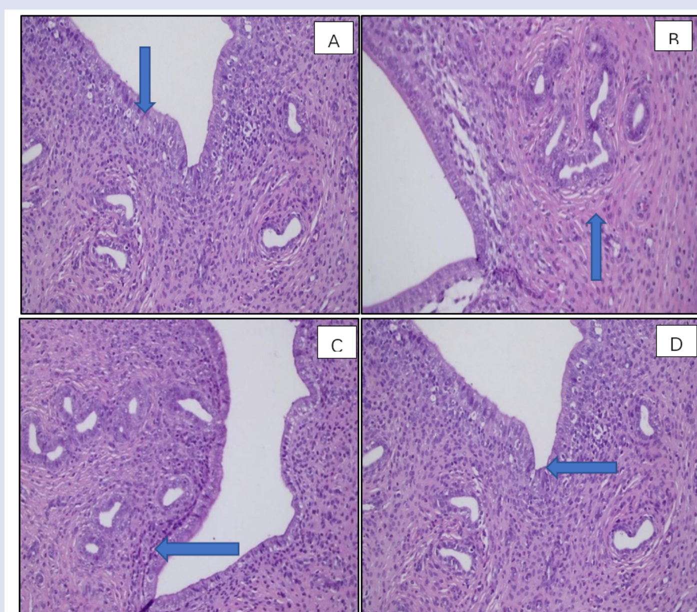

Figure 1: Histology of rat endometrial tissue throughout the estrus cycle, stained with H-E for histological dating. A, estrus, B met estrus, C diestrus, D proestrus. Magnification 200x
Vertical arrow A = Luminal epithelium, Vertical arrow B = glands, Horizontal arrow C = stroma, Horizontal arrow D = Lumen

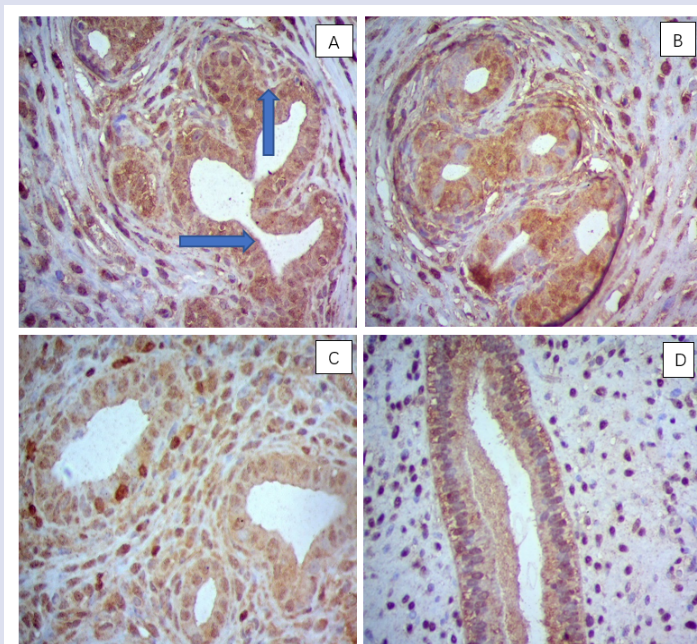

Figure 2: Immunohistochemical staining for EG-VEGF in rat endometrium. Their location is detected in the epithelial cells of the glands and stroma. A. Natural cycle, B, Stimulated cycle with a dose of 12.5 IU and C, Stimulated cycle with a dose of 25 IU, D positive control. Native magnification × 400. Horizontal arrow A = EG-VEGF expression in cell gland epithel, Vertical arrow = EG-VEGF expression in sroma cell.

free water. The cycling conditions were 95ºC for 5 min, 40 cycles with 95ºC for 5 seconds, 57ºC for 10 seconds, and 72ºC for 15 seconds. The qPCR was carried out on the Prime Pro 48 Real-Time PCR (Techne Cole-Parmer, Staffordshire, United Kingdom). Quantification was performed using an external standard curve with five serial dilutions between 10 and 10^{-3} ng/ul.

Primer sequence

Gene	Primer Sequence	
	Forward	Reverse
PROK1	AAG TCT TCA TCA TGC TCC TTC T	ACA CTG GAC ATC TCG TTC AC

Statistical analysis

Data are presented in mean +/-SD and were analyzed using one-way ANOVA for data with normal distribution followed by a post-hoc test. The value of $p < 0.05$ indicated a significant difference. The overall statistical analysis of this study was conducted using IBM SPSS 22 software.

RESULT

Endometrial dating

Histological dating of the endometrium was assessed on the basis of changes occurring in the endometrial luminal epithelium (18). In the estrus phase, the epithelium lining the lumen changes from low columnar, during metestrus and diestrus to columnar high in proestrus and estrus. A total of 36 rat endometrial samples were assessed, 27 samples in the secretory phase and one sample in the proestrus period (figure 1).

Distribution and quantification EG-VEGF

Distribution and cellular quantification of EG-VEGF protein was assessed by comparing the intensity of the brown color that appeared in each immunohistochemical preparation. A total of 27 samples were tested positive with varying intensity of brown color, ranging from weak to very strong intensity. The intensity of the brown color in the glandular compartment is stronger than in the stroma. Furthermore, the expression of EG-VEGF in the glandular and stromal compartment of the endometrium, the intensity of the brown color that appears is stronger in the control group compared to the stimulated group (Figure 2).

EG-VEGF expression in all CPI images was calculated using the Histo Score (H-Score) formula. The results can be seen in table 1. The results of the ANOVA test, that the expression of EG-VEGF in the endometrial glands showed a significant decrease from the normal cycle group to the stimulated cycle group 1 (SC 1) and SC2 (P = 0.00). EG-VEGF in the endometrial stroma (P = 000). Furthermore, the secretion of estadiol and progesterone hormones from rat serum taken on the first, second and third days after the estrus period did not show a significant decrease between the normal cycle group and the stimulated cycle group (P = 0.48 and P = 0.13).

The mean number of births of female rats in the control group and the treatment group at a dose of 12 IU which were mated with male rats, namely 10 and 4 in the treatment group at a dose of 25 IU (table 1). Statistical analysis, there was a significant decrease between the normal cycle group and SC 2 (P = 0.03), while the SC 1 group was not significantly different (P > 0.05).

mRNA gene expression analysis

The expression value of EG-VEGF mRNA using quantitative real-time PCR (qPCR) technique obtained very small expression results, even some samples were not detected for expression. The statistical test results did not show a significant difference in EG-VEGF mRNA expression between the natural cycle and the stimulated cycle (P > 005).

DISCUSSION

One of the important factors in the implantation process is the optimal level of endometrial receptivity during the implantation window period. In our previous study, we reported evidence that HOXA10 expression reduced after administration of hCG, which indicates that there has been a disruption in the reception of endometrium during implantation period.[57] In this study, we investigated one of protein molecules expressed in the luminal epithelium, which is the endocrine gland vascular endothelial growth factor (EG-VEGF). In the natural cycle, the development of the endometrium reaches a receptive stage in the pre-implantation period controlled by the ovarian hormone's estrogen and progesterone. Steroid hormone secretion which varies throughout the phases of the menstrual cycle has a significant effect on EG-VEGF production.[31,32] On the other hand, the supraphysiology of progesterone and estrogen during assisted reproductive treatment adversely affects endometrial receptivity, but the mechanism has not been fully elucidated. In addition, its correlation with pregnancy rates in IVF treatment has not been reported. Research in women following assisted reproductive procedures is difficult, bogged down with ethical issues, so we studied the endometrium in mice. The aim was to determine the effect of recombinant FSH administration on the expression of EG-VEGF protein (which has been proposed as a marker of endometrial receptivity) and its correlation with steroid hormone levels and the number of births obtained.

EG-VEGF expression in metosetroes phase was analyzed using immunohistochemical (IHC) and quantitative real-time PCR (qPCR) techniques. A total of 32 samples of the endometrium were examined, the locations of which were detected in the cytoplasm of glandular and stromal epithelial cells. Expression of EG-VEGF in both gland and stroma may play an important role in the development of the endometrium to reach the receptive stage in the pre-implantation period. Previous studies also reported that EG-VEGF expression levels were also detected in the gland and stromal compartments and their expression peaked in the mid-luteal phase.[24,33,34] Increased expression in the mid luteal phase shows its role at the time of embryo implantation.[35,36] According to Battersby, the expression level of EG-VEGF in the glandular epithelium was higher than in the stroma, suggesting that it may be synthesized by the glands and secreted into the stroma.[23] Poor receptivity of the endometrium in rat uterus in the pre-implantation period was also found in other markets such as Mucin-1 and leukemia inhibitory factor (LIF).[37-39] We have compared EG-VEGF expression levels in the endometrium of secretory phase mice that were higher in the cycle group). natural compared to the stimulated group. A significant decrease in expression in the stimulated group indicates that the conditions for endometrial receptivity are not optimal in receiving embryo implantation. It is known that the role of EG-VEGF during implantation of pregnancy, in addition to influencing embryonic embryo growth, also acts on acceptance through the process of angiogenesis, vascular permeability and increased adhesion of endometrial epithelial cells.[40-42] Increased expression of EG-VEGF in decidual tissue during early pregnancy functions to mediate fetal-maternal dialogue through regulation of LIF expression.[43]

In this study, we measured the expression levels of EG-VEGF in the endometrium of mice in the natural cycle group and the group stimulated with 12.5 IU and 25 IU recombinant FSH doses. All samples were assessed on the first day, second day and third day after the estrus phase in the natural cycle group and after administration of hCG in the stimulated cycle group. Our data show that EG-VEGF expression was higher in the endometrium of female mice in the natural cycle (SA) group compared to the cycle stimulated either at the stromal site or in the gland. Statistically, the average value of EG-VEGF expression on the first, second and third days there was a significant decrease from the natural cycle group to the stimulated cycle group (table 1). There is

a tendency that the administration of recombinant FSH, especially at a dose of 25 IU, affects the receptivity of the endometrium. During the COH procedure in the assisted reproductive program, gonadotropins are administered to stimulate the development of many mature oocytes to increase the success of IVF. As a consequence of the development of many follicles there is an increase in the concentration of steroids. Impact of supraphysiological steroid hormone concentrations However, we have previously shown that supraphysiological hormone concentrations significantly reduce implantation and pregnancy rates.[44] The low implantation rate is likely the result of peri-implantation endometrial gland-stromal asynchronous.[45] In the natural cycle, the development of the endometrium in the implantation phase is characterized by a tortuous glandular shape. The results of research by Battersby *et al*. showed that both EG-VEGF and its receptor are overexpressed in the secretory phase of the menstrual cycle.[23]

Our data show that there appears to be a correlation between the dose of recombinant FSH administered to the stimulated group and the level of EG-VEGF expression, particularly the 25 IU dose). A previous study also reported that the transcriptional level of EG-VEGF mRNA expressed in peri-implantation endometrium was significantly decreased in high ovarian responders compared to NC.[46] When associated with EG-VEGF function in the implantation process and early pregnancy, the decreased expression This receptivity marker will adversely affect the ability of the endometrium to accept embryo implantation. Previous studies reported that the non-optimal expression of EG-VEGF in the pre-implantation period can interfere with the embryo implantation process. Decreased EG-VEGF expression in both serum and follicular fluid has been reported in low pregnancy, recurrent miscarriage and in infertile patients.[47-49] In this study we obtained significantly decreased birth data between the natural cycle group and the SC 1 and SC2 cycles. (table 1). The average decrease in the number of births in the 25 IU (SC2) stimulated cycle group was above 50% compared to the natural cycle and SC 1 (table 1). These results indicate that there is a positive correlation between the dose level given to impaired endometrial receptivity, but successful implantation can be achieved due to the synergism between the embryonic developmental stage and the receptive endometrial environment.

Our data showed that there was considerable variation in EG-VEGF expression in both the stroma and glands, especially in the stimulated cycle group (Table 1). It is possible that its expression is controlled by the hormone estrogen and progesterone. Changes in steroid hormone levels due to the administration of ovarian stimulators have different effects on the development of the endometrium that are not synchronized according to the receptive phase. Asynchronous development of the endometrium during the receptive period of the endometrium during the CRH procedure has been widely reported. When endometrial development progresses for several days, so that it does not match the phase causing no pregnancy 25. In this study we found that steroid levels (estradiol and progesterone) increased in stimulated cycle group, although not significantly. High levels of steroids cause endometrial development to be out of sync with glandular maturation and stromal morphology. The results of previous studies have reported that the impact of high steroid increases during the COH procedure is debatable. Some studies show a negative impact, while others show a positive impact on ART outcomes and others show no impact.[50-54] According to Paulson (2011) supraphysiological elevation of serum estradiol impairs endometrial receptivity.[55] High estradiol concentrations can be associated with abnormal placentation because estradiol is the main hormone affecting endometrial growth and preparation for decidualization. Correlation between high estradiol concentrations and abnormal placentation by assessing the number and rate of pregnancy complications associated with abnormal placentation. Furthermore, a recent study by Healy *et al.* (2009) have found that obstetric bleeding caused by placenta previa and placental abruption is more common in IVF pregnancies and suggested that a possible mechanism is the effect of high estradiol concentrations on the endometrium at the time of implantation.[56-58]

Our data showed no significant difference between EG-VEGF mRNA expression in natural and stimulated cycles. Quantitative data obtained from these results are very small and even some samples are not detected so that it is difficult to analyze. The use of the relative assessment method is likely to get better results. In conclusion, decreased EG-VEGF expression in rat endometrium after recombinant FSH administration was associated with decreased endometrial receptivity which could decrease implantation rate.

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