ejpmr, 2017,4(4), 467-474

SJIF Impact Factor 4.161

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

EXPRESSION OF VASCULAR ENDOTHELIAL GROWTH FACTOR PROTEIN IN ENDOMETRIUM OF WOMEN WITH MENORRHAGIA

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Article Received on 02/02/2017 **Article Revised on 22/02/2017** **Article Accepted on 14/03/2017**

ABSTRACT

Objective: In the present study we tried to determine expression of Vascular Endothelial Growth Factor (VEGF) protein in endometrium of patients with menorrhagia. **Methods:** A total of 100 patients with history of menorrhagia were selected for study. Immunohistochemistry was performed on these endometrial biopsy sections to assess VEGF expression in endometrial glands, stroma and endothelium. Results: The results of VEGF expression were compared with phase of menstrual cycle. In late proliferative phase (p value =0.0461) and in late secretory phase (p value =0.0001) there was statistically significant association between VEGF protein expression on glandular luminal surface of endometrium in cases and controls (p value =0.0461). In late proliferative phase (p value =0.0461) and in late secretory phase(p value <0.0001) there was statistically significant association between VEGF protein expression in entire glandular cytoplasm of endometrial glands in cases and controls (p value =0.0461). The findings of the present study clearly observed that glandular luminal secretions were observed more in menorrhagia patients as compared to cytoplasmic positivity in non menorrhagic patients. **Conclusion:** There was abnormal localization of VEGF in glandular epithelium of menorrhagia patients.

KEYWORDS: Angiogenesis, Endometrium, Menorrhagia, VEGF.

INTRODUCTION

Menorrhagia is defined as a loss of greater than 80 ml of blood at the time of menstruation.[1] The term is derived from the Greek language and means "to burst forth monthly- (mene, 'the moon' and rhegnmi, 'to burst forth').[2] It is one of the most important cause of gynaecology referrals in women and accounts for almost 50% of all hysterectomies.[3] Recently attempts have been made to study the functional aspects of endometrium to elucidate the cause of menorrhagia. As these mechanisms are manifested at menstruation, understanding of these events in normal menstrual cycle is of utmost importance.[2]

Angiogenesis, defined as the process whereby new blood vessels are created from pre-existing vasculature, occurs periodically as part of the cyclical growth and shedding which takes place during menstrual cycle.[4] It involves a number of steps including activation of endothelial cells within the existing vessel, breakdown of the basement membrane, migration of the endothelial cell towards the stimulus, proliferation of the endothelial cells, fusion of two sprouts to form a continuous line of endothelial cells, tube formation and recommencement of blood flow.[5] Angiogenesis occurs at three different times during endometrial cycle; during menstruation for vascular bed repair, during the proliferative stage when there is rapid growth of the endometrium and during the secretory stage when spiral arterioles undergo growth and coiling.[6] There is emerging evidence that abnormal angiogenesis may contribute to several endometrial related pathologies which not only includes endometrial cancer but also endometriosis, menorrhagia and breakthrough bleeding.[4] Vascular endothelial growth factor (VEGF), is one of the most important growth factor involved in angiogenesis. It is a potent endothelial cell mitogen which increases vascular permeability and plays a central role in inflammation and other pathologies. VEGF is expressed in a wide range of cells and tissues including rodent, primate and human endometrium.[4] Various isoforms of VEGF are now recognized and several studies have related VEGF expression in human endometrium to stages in the menstrual cycle.[7]

There is disagreement regarding VEGF expression in the human endometrium during different stages of menstrual cycle but most authors believe that VEGF m-RNA expression is low in proliferative phase, increases during late secretory phase and is maximum during menses.

However, no such clear pattern has been obtained in studies detecting VEGF protein expression, though expression was greater in glands than in stroma.[8] VEGF expression has been observed in endometrial blood vessels but this is further complicated by the expression of different isoforms of VEGF in different types of endometrial blood vessels.[7]

Though VEGF is considered fundamental to endometrial angiogenesis, details of how and when different endometrial cell types produce VEGF, and how production and activity is controlled by estrogen and progesterone still remains to be elucidated. Evidences suggest that different splice variants of VEGF may have a role in regulating angiogenesis at a local level.[4] The fact that vascular repair is an obvious feature of menstrual cycle does not necessary imply that disturbance of angiogenesis results into menorrhagia but certain evidences suggest that altered VEGF expression may be involved in pathogenesis of menorrhagia. Increased fibrinolytic activity and enhanced vasodilation are the two hypotheses used to explain heavy periods. VEGF is known to stimulate tissue plasminogen activator, a potent fibrinolytic and nitrous oxide, a vasodilator.[3] Increased immunoreactivity for VEGF has been detected in women with menorrhagia[3] and also in peritoneum of women suffering from endometriosis.[9]

The objectives of present study were to study VEGF protein expression in endometrium and determine if there was any significant difference in VEGF protein expression in women with menorrhagia as compared to women with normal menstrual cycles stratified by phase of menstrual cycle and cell type. In this study we examined the expression and distribution of VEGF protein by immunohistochemistry.

The present study plans to evaluate if there was any alterations in angiogenic factors and angiogenesis in patients of menorrhagia so that drugs targeted against these factors may be used as medical treatment in this common problem affecting large number of women in reproductive age group.

The hypothesis of the current study was that in women menorrhagia will have elevated VEGF protein expression in endometrium as compared to age matched women with normal menstrual cycles. There will be abnormal localization of VEGF protein expression in endometrium of women with menorrhagia and VEGF will be expressed more in proliferative phase. VEGF protein expression will be more in stratum functionalis as compared to stratum basalis.

MATERIAL AND METHODS

This study was carried out in, a rural teaching hospital in Central India. All women admitted to the Gynecology inpatient ward were screened to identify the cases that satisfied the following inclusion criteria: Age between 20 and 45 years, History of menorrhagia with duration of symptoms for 3 months or more, hysterectomy was planned as a standard therapeutic procedure, in patients of menorrhagia who did not respond to previous conservative therapy.

Patients excluded from the study were: if no hysterectomy could be performed due to peri-operative complications, any pathology was determined in the hysterectomy specimens such as gynecological malignancy, endometritis, adenomyosis, fibroids, any systemic cause of menorrhagia was evident such as thrombocytopenia, sepsis, etc. Controls satisfied the following inclusion criteria: Age between 20 and 45 years, age matched with cases, history of primary or secondary infertility but normal menstrual cycles, endometrial biopsy was planned as a standard diagnostic procedure, for evaluation of infertility or endometrial biopsies taken from healthy fertile women undergoing tubal ligation or hysterectomy for prolapse.

A written informed consent was sought to carry out additional tests on the endometrial samples. Hysterectomy and endometrial biopsy specimens were fixed in 10% buffered formalin and processed through wax by routine histological techniques and subsequently stained with haematoxylin and eosin[9] and also kept for immunohistochemistry and dating of collected endometrial specimens by Noyes et al[10] specific criteria.

Immunohistochemistry determination of VEGF protein[11-15] was detected by using a rabbit anti-human polyclonal anti-VEGF antibody. Sections boiled for 10 minutes in 10mmol/sodium citrate buffer for antigen retrieval followed by quenching of endogenous peroxidase activity, and then blocking with 10% goat serum. The primary antibody (Polyclonal, Immunogen: Human recombinant VEGF 165, Clone: Polyclonal, Species: Rabbit, Protein Conc.: 10-15mg/ml, Catalog No. PU483-UP, BioGenex, USA) was then be applied overnight at 2-4^{o}C, followed by sequential incubations with biotinylated secondary antibody, streptavidin-HRP conjugate and DAB chromogen. Positive and negative controls were included in each staining run.

VEGF staining was scored in each compartment like stratum basalis and functionalis, endometrial glandular epithelium, stroma and endothelium/vessels.(16) Staining in each compartment was scored according to two criteria: the degree of staining (0 = no staining, 1 = 1/250 cells stained, 2 = 1/50 cells stained, 3 = 1/10 cells stained and 4 > 1/10), and the intensity of staining (0 = no staining, 2= moderate, 3 = intense) .These score were then multiplied to obtain immunohistochemical staining score. These score were later graded as Grade 0 = Score 0, grade 1= 1 to 4, Grade 2 = 5 to 8 and Grade 3 = 9 to 12.

RESULTS

A total of 100 cases and 100 controls were evaluated in present study. We performed a descriptive statistical

analysis of collected variables and compared expression of Vascular Endothelial Growth Factor (VEGF) Protein in endometrium of women with menorrhagia with phase of menstrual cycle, endometrial cell type, endometrial cell layer. We used statistics software EPI-6 for all statistical analysis. Chi square test was applied and p value was calculated.

The age of the patients in this study ranged from 25 years to 45 years. Maximum number of patients (69%) were in the age group of 40-45 years followed by 24% in 35-39 years, 5% in 30- 34years and 2% in 25-29 years respectively. Controls were age matched with patients. Out of 100 cases 84 were in proliferative phase, 13 were in secretory phase and 3 in menstruating phase. In controls 19 were in proliferative phase, 81 were in secretory phase.

The dating of endometrium was done in all cases and controls. Most of the cases were in proliferative group whereas most of controls were in secretory group. The main limitation of present study was large number of controls were in late secretory phase of cycle. This was inherent problem in study because most of controls available were females being investigated for infertility and the investigation was mainly done to demonstrate ovulation.

In our study the staining reaction was stronger in stratum functionalis as compared to stratum basalis. Stratum basalis could be examined in only cases as control specimens were obtained by endometrial biopsies. Most of these biopsies either did not show stratum basalis or it was very scanty for immunohistochemistry interpretation.VEGF protein expression was examined in glandular epithelium, endometrial stroma and endothelium in endometrium. In glandular epithelium, VEGF protein expression was seen in two distinct forms, one was luminal surface positivity and second was positivity seen in entire epithelial cytoplasm. VEGF protein expression on glandular luminal surface of endometrium was looked for any variations with reference to the phase of menstrual cycle. During proliferative phase the luminal surface positivity for VEGF was seen in statistically significant number of cases as compared to controls. In late proliferative phase there was statistically significant association between glandular luminal surface VEGF protein expression in cases and controls (p value =0.0461). In late secretory phase there was highly statistically significant association between glandular luminal surface VEGF protein expression in cases and controls (p value =0.0001). Just opposite findings were noted in glandular cytoplasmic VEGF positivity as shown in Table No.1.

VEGF protein expression in entire glandular cytoplasm of endometrial glands was looked for any variations with reference to the phase of menstrual cycle. In late proliferative phase there was statistically significant association between glandular cytoplasmic VEGF protein expression in cases and controls (p value =0.0461). In late secretory phase, there was highly statistically significant association between glandular cytoplasmic VEGF protein expression in cases and controls (p value <0.0001). We also observed that luminal positivity was consistently seen after mid proliferative phase in patients with menorrhagia except early proliferative phase. In early proliferative phase both luminal surface and cytoplasmic positivity was not seen in any case as well as controls. We determined VEGF protein expression in glandular elements with reference to endometrial layer in cases and controls. The difference in proportion of glandular cytoplasmic and luminal VEGF positivity in cases and controls in stratum functionalis were statistically significant (P< 0.01) as shown in Table No.2.

VEGF expression with reference to endometrial cell type was also assessed, the difference between VEGF protein expression on glandular luminal surface in endometrium and glandular cytoplasm of endometrium between cases and controls was found to be statistically highly significant (P< 0.001). VEGF protein expression in stromal (P=0.001) and endothelial cells (P<0.001) of endometrium between cases and controls was statistically highly significant (P< 0.001) as shown in Table No.3.

When the findings of VEGF positivity was assessed together for luminal surface VEGF positivity and cytoplasmic positivity it was observed that luminal surface positivity was predominantly seen in cases in both proliferative as well as secretory phase as compared to controls which showed mainly cytoplasmic positivity in both proliferative and secretory phase as shown in Table No.4. The difference of proportion of glandular luminal surface and cytoplasmic VEGF positivity among the cases and controls were statistically insignificant (p value > 0.05). When grading of glandular luminal surface VEGF positivity in cases and controls (p< 0.001)were done, there was highly statistically significant association in secretory phase whereas in proliferative phase the difference was not statistically significant.

When grading of glandular cytoplasmic positivity for VEGF in cases and controls were done, there was highly statistically significant association in proliferative (p value=0.0055) as well as secretory phase (p value <0.0001). On grading glandular cytoplasmic VEGF positivity in cases and controls it was observed that in cases grade 2 & 3 positivity was seen only in secretory phase. Similarly grade 2 and grade 3 positivity was also observed in controls in secretory phase mainly late secretory phase.

When grading of endothelial cells positivity for VEGF was done ,we observed that in early proliferative phase most of the cases were negative, in mid proliferative phase there were cases showing grade 1 positivity and in

late proliferative phase more cases showed grade I positivity. In some cases there was grade 2 positivity. In secretory phase all cases were showing expression and in late secretory phase there was grade 3 expression in cases. Similar findings were also noticed in controls and there was no significant association of grading of endothelial cells positivity in cases as well as controls.Similar observations were also seen when grading of stromal cells positivity for VEGF in cases and controls.

Table No.1: Glandular cytoplasmic VEGF positivity with phase of menstrual cycle.

Phase of Menstrual Cycle	Case No.	VEGF (Cytoplasmic positivity) No.	Control No.	VEGF (Cytoplasmic positivity) No.
Early Proliferative	14	0(0%)	2	0(0%)
Mid Proliferative	17	0(0%)	2	1(50%)
Late Proliferative	53	0(0%)	15	2(13.3%)
Early secretory	5	2(40%)	0	0(0%)
Mid secretory	2	1(50%)	0	0(0%)
Late secretory	6	0(0%)	81	80(98.7%)
Menstruating	3	0(0%)	0	0(0%)

Table No.2: VEGF expression in glandular elements of stratum functionalis

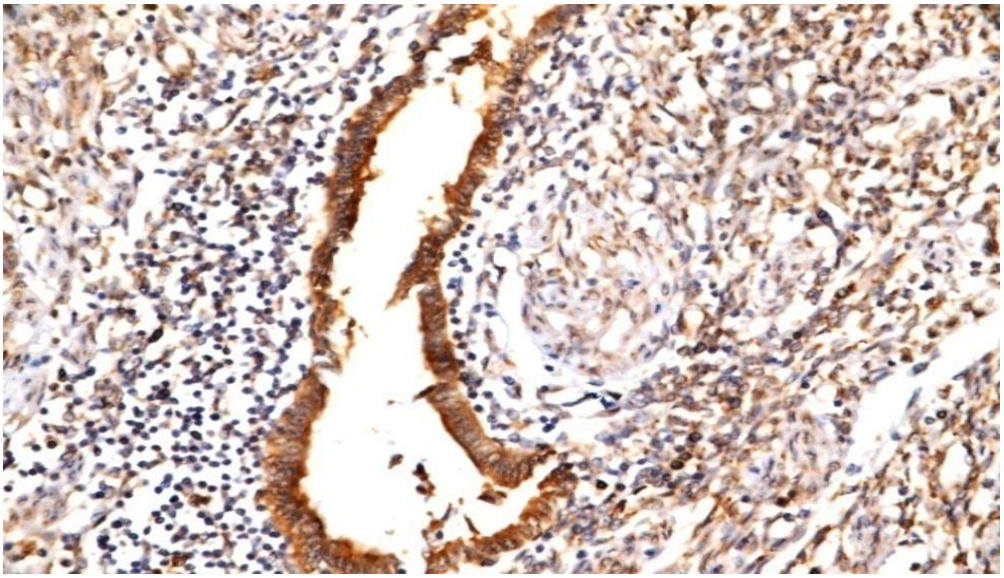
Location	Cases/Control	Positive No. (%)	Negative No. (%)	Total No. (%)
Glandular cytoplasmic	Case	3 (3%)	97 (97%)	100 (100%)
	Control	83 (83%)	17 (17%)	100 (100%)
Glandular luminal	Case	83 (83%)	17 (17%)	100 (100%)
	Control	15 (15%)	85 (85%)	100 (100%)

Table No. 3: VEGF expression with reference to endometrial cell type.

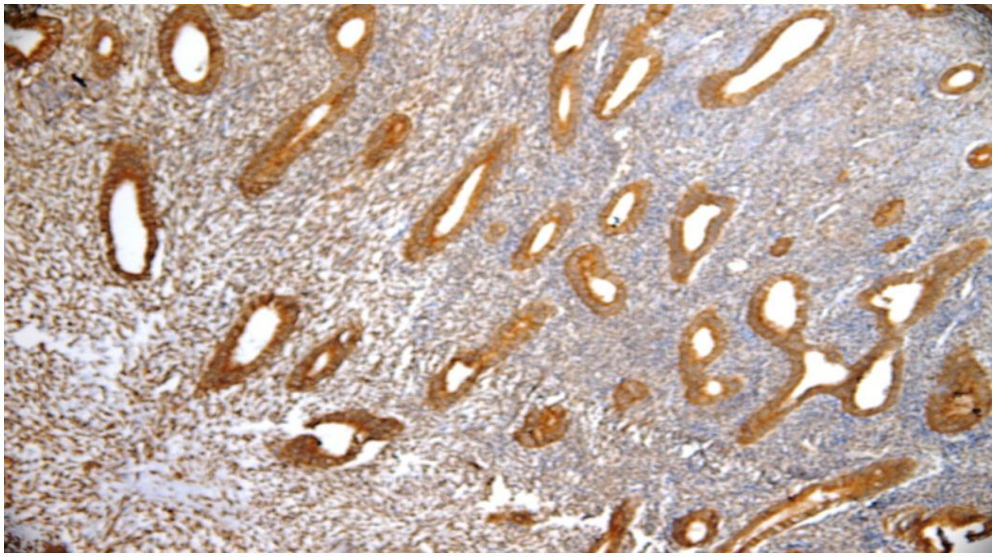
Endometrial cell type	Cases		Control	
	Positive No. (%)	Negative No. (%)	Positive No. (%)	Negative No. (%)
Glandular LuminalSurface VEGF	83 (83%)	17 (17%)	15 (15%)	85 (85%)
Glandular Cytoplasmic VEGF	03 (03%)	97 (97%)	83 (83%)	17 (17%)
Stromal cells VEGF	80 (80%)	20 (20%)	95 (95%)	05 (05%)
Endothelial cell VEGF	78 (78%)	22 (22%)	95 (95%)	05 (05%)

Table No. 4: Glandular VEGF positivity with phase of menstrual cycle.

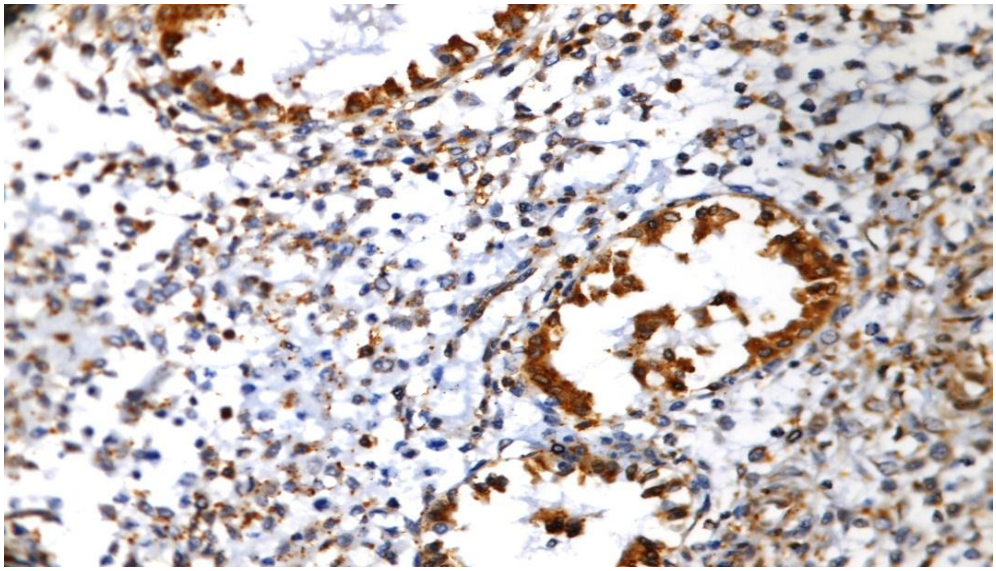
Phase of Menstrual Cycle	Case No. (%)	VEGF luminal surface positivity No. (%)	VEGF cytoplasmic positivity No. (%)	Control No. (%)	VEGF luminal surface positivity No. (%)	VEGF cytoplasmic positivity No. (%)
Early Proliferative	14	0(0%)	0(0%)	2	0(0%)	0(0%)
Mid Proliferative	17	17(100%)	0(0%)	2	1(50%)	1(50%)
Late Proliferative	53	53(100%)	0(0%)	15	13(86%)	2(14%)
Early secretory	5	3(60%)	2(40%)	0	0(0%)	0(0%)
Mid secretory	2	1(50%)	1(50%)	0	0(0%)	0(0%)
Late secretory	6	6(100%)	0(0%)	81	1(1.2%)	80(98.8%)
Menstruating	3	3(100%)	0(0%)	0	0(0%)	0(0%)



Photomicrograph 1.



Photomicrograph 2



Photomicrograph 3

Figure legends

Photomicrograph 1 is showing VEGF expression in late proliferative phase with glandular luminal surface positivity with stromal and endothelial positivity (IHC x 40)

Photomicrograph 2 is showing VEGF expression in mid proliferative phase with glandular luminal surface positivity in both stratum basalis and stratum functionalis with stromal and endothelial positivity in functionalis layer (IHC x 40)

Photomicrograph 3 is showing VEGF expression in late secretory phase with glandular cytoplasmic positivity with stromal and endothelial positivity (IHC x 40)

DISCUSSION

In our study, glandular epithelium of endometrium, VEGF protein expression was seen in distinct two forms, one was luminal surface positivity and second was positivity seen in entire epithelial cytoplasm. **VEGF-A** expression is both cell specific and cycle specific. Charnock Jones et al in 1993[17]; Li et al in 1994[18]; Shifren et al in 1996(9) in their studies using Immunohistochemistry and in-situ hybridization demonstrated VEGF-A in the luminal and glandular epithelium in the proliferative phase of the cycle**.**

In our study VEGF protein expression on glandular luminal surface of endometrium was looked for any variations with reference to the phase of menstrual cycle. VEGF positivity on the glandular luminal surface was absent in both cases and controls in early proliferative phase. In the mid and late proliferative phase all cases showed positivity at luminal surface as showm in photomicrograph 1 &2. However in controls it was observed in 50% of controls in mid proliferative phase and 86% of late proliferative phase. Similarly in early and mid secretory phase many cases showed luminal surface VEGF expression. All cases showed luminal surface VEGF positivity in late secretory and menstruating phase as compared to only one out of 81 controls. The findings were statistically significant.

Just opposite findings were noted in glandular cytoplasmic VEGF positivity. Here none of the cases were positive in proliferative phase as well as late secretory and menstruating phase as compared to almost all controls in late secretory phase were positive for VEGF expression in cytoplasm as shown in Table No.1 & photomicrograph 3.

Torry & Torry (1997)[19] and Rogers & Gargett (1999)[20] in their study observed that bulk of endometrial VEGF was glandular and 80% of this VEGF was secreted from luminal surface. Hornung et al in 1998[21] also observed that in both primates and rodents ,most of the epithelial VEGF was secreted apically into the uterine lumen. Charnock-jones et al in 1993[17], Shifren et al in 1996 (9), Torry et al in 1996[22], Graubert et al in 1997[23] in their mRNA studies done with in-situ hybridization and northern blots observed that VEGF expression in whole endometrium was low during the proliferative phase, increased during the late secretory phase, and reached the maximum at menses probably which reflected glandular expression. Shifren et al in 1996 (9) found 1.6 fold , 2.0 fold and 3.6 fold increase in VEGF mRNA levels in tissue taken in the mid and late proliferative and secretory phases of the cycle respectively. Surprisingly, the most intense immunoreactivity for VEGF was found in glandular epithelium of hypogonadal animals.[24]

The findings of the present study clearly observed that glandular luminal secretions were observed more in menorrhagia patients as compared to cytoplasmic positivity in non menorrhagic patients. It may also be responsible for difference in observation made in different studies as most of the studies have not taken into account positivity of luminal surface or entire cytoplasm. There are only few studies which have taken this into account and none of them were done in menorrhagia patients. We also observed that luminal positivity was consistently seen after mid proliferative phase in patients with menorrhagia except early proliferative phase. It was also observed from present study that in early proliferative phase both luminal surface and cytoplasmic positivity was not seen in any case as well as controls. Gargett et al in 1999[8] have observed elevated VEGF staining in early proliferative phase. Our findings suggested that whatever role VEGF may play in the angiogenesis of endometrium is important except in early proliferative phase.

Malik et al in 2006[2] foundthatthe concentration of VEGF-A in the effluent of women with an menstrual blood loss (MBL)>80 ml was significantly lower (P<0.002). The levels of mRNA encoding VEGF-A in the menstruated endometrium from women with menorrhagia was significantly reduced.

Mints et al 2007(25) found that the number of vessels that expressed VEGF-A, VEGFR-1, or VEGFR-2 was also significantly greater in patients with Idiopathic menorrhagia than in controls, and gap size correlated significantly with vascular expression of VEGF-A and VEGFR-1. VEGF expression with reference to endometrial cell type was also assessed in present study. On grading of endothelial cells positivity for VEGF we observed that the grade increased in cases along with progression of the cycle. Similar findings were also noticed in controls and there was no significant association of grading of endothelial cells positivity in cases as well as controls.

Similar observations were also seen when grading of stromal cells positivity for VEGF in cases and controls. It was observed that with progression of cycle there was more often and stronger expression of VEGF in cases and controls. In 1993, Charnock-Jones et al[17] showed that mRNA encoding for VEGF was present in epithelial

and stromal cells within the endometrium during the proliferative phase.

Mints et al in 2005[26] in his study found that the number of capillaries that stained for VEGF-A, VEGFR-1, and VEGFR-2 was significantly higher in the menorrhagia group compared with controls and suggested that VEGF-A, VEGFR-1, and VEGFR-2 were more actively expressed in capillaries in menorrhagic endometrium.

Gargett et al in 1999[8] in his study found that *in vivo*, VEGF was significantly elevated during the early proliferative phase of the menstrual cycle. However, no relationship was observed between in-vitro VEGF production by cultured endometrial explants, glandular epithelial or stromal cells and the cycle stage. Two other studies by (Li et al in 1994 and Lau et alin 1999)[18,27] used large sample numbers and demonstrated a trend to higher stromal VEGF concentrations during the proliferative stages ,although study by Shifren et al in 1996[9] of unknown sample number demonstrated increasing VEGF mRNA and protein from early proliferative to late secretory endometrium.

Charnock-jones et al in 1993[17], Torry and Torry in 1997[19] in their study observed that during the proliferative phase of the menstrual cycle, VEGF was expressed by both the epithelial and stromal endometrial cells, but in the secretory phase it was expressed only by epithelial cells. While this may suggest that VEGF expression was hormonally regulated, VEGF expression by endometrial cells in response to steroids was inconsistent, and hormones may not be the major regulators. There was disagreement regarding correlation of VEGF expression in the human endometrium to stages of menstrual cycle but most authors in their mRNA studies (in-situ hybridization and Northern blots) observed that VEGF m-RNA expression was low in proliferative phase, increases during late secretory phase and was maximum during menses. However, no such clear pattern was obtained in studies detecting VEGF protein expression, though expression was greater in glands than in stroma.[18,9,27] Shifren etal in 1996[9] demonstrated increasing VEGF mRNA and protein from early proliferative to late secretory endometrium. The stromal and endothelial VEGF expression was comparatively higher in controls than menorrhagia patients. Torry et al in 1996[22] found a 3- fold to 6- fold increase in endometrial mRNA levels encoding VEGF in the secretory phase of the menstrual cycle.

The conclusions drawn from present study were abnormal localization of VEGF in glandular epithelium of menorrhagia patients. In controls as well as cases the VEGF was initially localized on luminal surface but in secretory phase, controls showed localization of VEGF throughout cytoplasm, where as in menorrhagia patients it predominantly continued to be localized at luminal surface. The stromal and endothelial VEGF expression was comparatively higher in controls than menorrhagia patients.It was observed that with progression of cycle there was more often and more stronger expression of VEGF in cases and controls.

We could study all the components of hypothesis. This study only looks at small part in process of angiogenesis. We need to study role of other cytokines, female sex hormones which regulate menstrual cycles. We also need to evaluate VEGF mRNA expression and compare it with abnormal localization of VEGF observed in present study before confirming abnormal angiogenesis to be cause of excessive menstrual bleeding. However, in spite of the limitation we were able to demonstrate abnormal localization of VEGF in endometrial glands in patients of menorrhagia, as well as we were able to demonstrate increased endothelial cell proliferation in patients in the premenstrual part of cycle.

ACKNOWLEDGMENTS

The authors thank Dr Nitin Gangane for histopathology work and technical staff of Department of Pathology, MGIMS Sewagram and Department of obstetrics and Gynaecology for hysterectomy and endometrial samples.

DISCLOSURE: The authors state that there are no conflicts of interest to disclose.

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