

## Original Research Article

# Correlation of endothelial cell proliferation with vascular endothelial growth factor in endometrium of women with menorrhagia

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## ABSTRACT

**Background:** Approximately 30% of women of reproductive age experience excessive blood loss during menstruation. In 50% of cases, menorrhagia has no underlying pathology. However, until recently, the only permanent cure for menorrhagia was hysterectomy. In this study we aim to determine the correlation of vascular endothelial growth factor (VEGF) expression with markers of endometrial endothelial cell proliferation like proliferating cell nuclear antigen (PCNA) and Cluster Determination (CD34).

**Methods:** A total of 100 patients with history of menorrhagia were selected for study. Double Immunohistochemistry was performed on these endometrial biopsy sections. Proliferating endothelial cells were identified by an immunohistochemical double staining technique with PCNA and CD34. VEGF expression was also seen in endometrial biopsy.

**Results:** In general, expression of both VEGF and PCNA was more in functional layer than basal layer in both menorrhagic patients as well as non menorrhagic patients. When glandular cytoplasmic VEGF expression was compared with PCNA the association was statistically significant whereas completely opposite findings was seen with glandular luminal surface VEGF positivity but the association was statistically significant. In secretory phase (p-value<0.001) there was highly statistically significant association in PCNA grading with glandular luminal surface VEGF positivity whereas when we correlated PCNA with cytoplasmic glandular VEGF in secretory phase it was statistically significant (p-value<0.001).

**Conclusions:** The endothelial proliferation was significantly higher in menorrhagia patients during late secretory phase of cycle than controls. We were able to demonstrate increased endothelial proliferation in patients in the premenstrual part of cycle.

**Keywords:** Angiogenesis, Endometrium, Endothelial cells, Menorrhagia, VEGF

## INTRODUCTION

Menorrhagia is defined as a loss of greater than 80 ml of blood at the time of menstruation.<sup>1</sup> The term is derived from the Greek language and means “to burst forth monthly- (mene, ‘the moon’ and rhegnmi, ‘to burst forth’).<sup>2</sup> It is one of the most important cause of gynaecology referrals in women and accounts for almost 50% of all hysterectomies.<sup>3</sup> The cause of menorrhagia

remains poorly understood as it is not associated with gross histological abnormalities of endometrium or that of hypothalamic-pituitary-ovarian axis and hence the only treatment option left in most cases is hysterectomy. 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.<sup>2</sup>

Normal menstrual cycle involves tissue breakdown, resulting in blood loss from damaged tissue followed by tissue repair. 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.<sup>4</sup> 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.<sup>5</sup> 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.<sup>6</sup>

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.<sup>4</sup> The process of angiogenesis is complex and though growth and differentiation of the endometrium and its vasculature is under the control of the hormones estrogen and progesterone, the sex steroids exert their effect through a variety of growth factors. 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.<sup>4</sup> Various isoforms of VEGF are now recognized and several studies have related VEGF expression in human endometrium to stages in the menstrual cycle.<sup>7</sup>

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.<sup>3</sup> Increased immunoreactivity for VEGF has been detected in women with menorrhagia and also in peritoneum of women suffering from endometriosis.<sup>3,8</sup> On the other hand, Malik S et al found lower VEGF-A concentration in menstrual effluent of women with menorrhagia when compared with controls.<sup>2</sup> They also found lower m-RNA expression of VEGF-A in desquamated endometrium of the same women. In this study, we aim to determine the correlation of VEGF expression with markers of endometrial endothelial cell proliferation like PCNA and CD34. The hypothesis of the current study is VEGF will be expressed more in

proliferative phase in endometrium of women with menorrhagia, VEGF protein expression will be more in stratum functionalis as compared to stratum basalis, endothelial cell proliferation will be seen in patients of menorrhagia.

## 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 and also kept for immunohistochemistry and dating of collected endometrial specimens by Noyes et al specific criteria.<sup>9,10</sup>

Proliferating endothelial cells was detected by a standard double immunohistochemistry protocol using mouse anti-rat antibody to proliferating cell nuclear antigen (PCNA) and a mouse monoclonal antibody against the cluster determination CD-34 antigen.<sup>11,12</sup> A sequential protocol was used with anti PCNA as the first primary antibody. Both primary antibodies was applied for 2-3 hours at 37° C, followed by sequential incubations with biotinylated secondary antibody. A streptavidin- Horse radish peroxidase (HRP) conjugate and Diaminobenzidine (DAB) chromogen was used for PCNA staining and a streptavidin-alkaline phosphatase conjugate with Nitro-blue tetrazolium/ 5-bromo-4-chloro-3'- indolyphosphate (NBT/BCIP) for CD- 34 staining. Endogenous peroxidase was quenched with 3% hydrogen-peroxide in 50% methanol. Positive and negative controls were included in each staining run. At least 100 microvessels

were counted in sequential fields scanned at X400 magnification from the surface epithelium through the full depth of section.<sup>13</sup> The number of these vessels containing proliferative endothelial cells were determined and the percentage of microvessels containing proliferating endothelial cells were calculated.

Grade 0: No PCNA positive endothelial cells in CD 34 positive blood vessels.

Grade 1: 1 to 10 % PCNA positive endothelial cells in CD 34 positive blood vessels

Grade 2: 11 to 50 % PCNA positive endothelial cells in CD 34 positive blood vessels

Grade 3: 51 to 100% PCNA positive endothelial cells in CD 34 positive blood vessels

Immunohistochemistry determination of VEGF protein.<sup>14-18</sup> 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°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/vessel: 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 scores were then multiplied to obtain immunohistochemical staining score. These scores were later graded as Grade 0 =Score 0, grade 1=1 to 4, Grade 2 =5 to 8 and Grade 3=9 to 12.<sup>19</sup>

## 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 and correlation of PCNA in CD34 positive blood vessels with VEGF expression. 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.

VEGF protein expression was examined in glandular epithelium, endometrial stroma and endothelium. 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.

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. 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. When we did grading of PCNA positivity, it was observed that there was no significant association of grading of PCNA positivity in CD 34 positive blood vessels in both proliferative and secretory phase.

Correlation of VEGF protein expression in cytoplasm of endometrial glands with PCNA positivity showed that when there was PCNA positivity, then more non menorrhagic patients had VEGF positivity as compared to menorrhagic patients which were mostly negative. The association was found to be statistically highly significant (p value <0.001). There was completely opposite findings for glandular luminal surface positivity. The correlation of VEGF protein expression on glandular luminal surface of endometrium with PCNA positivity showed that when there was PCNA positivity, then more menorrhagic patients had VEGF positivity as compared to non menorrhagic patients. The association was found to be statistically highly significant (p value <0.001)

We did the grading for PCNA and VEGF expression in the glandular elements of endometrium. When we correlated grading of PCNA expression with grading of VEGF expression on glandular luminal surface in menorrhagic and non menorrhagic patients during proliferative phase was not significantly associated and secretory phase (p value=0.0004128), it was highly significantly associated as shown in Table 1 and 2. On correlating grading of PCNA expression with grading of VEGF expression in cytoplasm of endometrial glands in menorrhagic and non menorrhagic patients during secretory phase, it was observed that the association was statistically significant (p value=0.005223) as shown in Table 3 and during proliferative phase p value could not be calculated (Test of significance could not be applied). VEGF expression with reference to endometrial cell type was also assessed in present study. The difference

between VEGF protein expression on glandular luminal surface in endometrium and glandular cytoplasm of endometrium between menorrhagic and non menorrhagic patients was found to be statistically significant (P<0.001). VEGF protein expression in stromal (P=0.001) and endothelial cells (P<0.001) of endometrium between menorrhagic and non menorrhagic

patients was statistically significant (P<0.001). When we did grading of endothelial cells and stromal cells positivity for VEGF there was no statistically significant association in proliferative as well as secretory phase and observed that with progression of cycle there was more often and more stronger expression of VEGF in menorrhagic and non menorrhagic patients.

**Table 1: Correlation of PCNA grading with glandular luminal surface VEGF grading in cases and controls during proliferative phase.**

Grading of PCNA	Grading of glandular luminal surface cytoplasmic VEGF									
	Cases					Controls				
	0	1	2	3	Total	0	1	2	3	Total
0	12	01	02	00	15	02	00	00	00	2
1	02	09	21	16	48	02	01	01	02	6
2	00	02	06	12	20	01	00	02	08	11
3	00	00	01	00	01	00	00	00	00	0

**Table 2: Correlation of PCNA grading with glandular luminal surface VEGF grading in cases and controls during secretory phase.**

Grading of PCNA	Grading of glandular luminal surface VEGF									
	Cases					Controls				
	0	1	2	3	Total	0	1	2	3	Total
0	00	00	00	02	2	02	00	00	00	2
1	02	00	01	01	4	09	00	00	00	9
2	01	00	01	05	7	68	01	00	00	69
3	00	01	01	01	3	01	00	00	00	1

p value = 0.0004128

**Table 3: Correlation of PCNA grading with glandular cytoplasmic VEGF grading in cases and controls during secretory phase**

Grading of PCNA	Grading of glandular cytoplasmic VEGF									
	Cases					Controls				
	0	1	2	3	Total	0	1	2	3	Total
0	02	00	00	00	2	00	00	01	01	2
1	02	00	00	02	4	00	00	01	08	9
2	06	00	01	00	7	01	03	28	37	69
3	03	00	00	00	3	00	00	00	01	1

**DISCUSSION**

In present study when we did grading of PCNA positivity, it was observed that in most menorrhagic patients and all controls in early proliferative phase PCNA was not expressed in blood vessels. But in later phases of cycle i.e. mid and late proliferative as well as secretory phase most of menorrhagic patients as well as controls showed expression except in menstruating phase. In present study, we observed that along with progression of cycle there was more expression of PCNA in CD34 positive blood vessel in both cases and controls. However, the association was not statistically significant

in both menorrhagic and non menorrhagic patients. Studies on endometrial endothelial cell proliferation was done by Kooy et al, Rogers, Gargett et al using endometrial endothelial cell proliferation as a measure of angiogenesis.<sup>5,13,19</sup> To identify proliferating endothelial cells, a immunohistochemical double staining system was used Goodger (Macpherson) and Rogers.<sup>20</sup> On grading of endothelial cells positivity for VEGF we observed that the grade increased in menorrhagic patients along with progression of the cycle. We found in early proliferative phase most of the menorrhagic patients were negative, in mid proliferative phase there were menorrhagic patients showing grade 1 positivity and in late proliferative phase more menorrhagic patients showed grade I positivity. In

some menorrhagic patients there was grade 2 positivity. In secretory phase, all menorrhagic patients were showing expression and in late secretory phase there was grade 3 expression in menorrhagic patients. Similar findings were also noticed in controls. Similar observation was also seen when grading of stromal cells positivity for VEGF in menorrhagic patients and controls. It was observed that with progression of cycle there was more often and more stronger expression of VEGF in menorrhagic patients and controls.

When glandular cytoplasmic VEGF expression was compared with PCNA it was observed that more number of controls were positive for VEGF and PCNA simultaneously as compared to controls which were negative for VEGF and positive for PCNA and association was also statistically significant.

We found completely opposite findings for glandular luminal surface positivity. Here we observed more number of positivity for PCNA as well as VEGF for menorrhagic patients as compared to controls which were mostly negative and the association was statistically significant.

When we compared PCNA grading with glandular luminal surface VEGF positivity in secretory phase, there was highly statistically significant association in both menorrhagic patients as well as controls whereas when we correlated PCNA with cytoplasmic glandular VEGF in both proliferative and secretory phase test for significance could not be applied in proliferative phase whereas in secretory phase it was statistically significant.

The study by Kooy et al 1996 and Goodger (Macpherson) and Rogers showed that there were no significant peaks in endothelial cell proliferation across the menstrual cycle.<sup>19,20</sup> Kooy et al demonstrated that endothelial cell proliferation was doubled in women with menorrhagia. Indeed, a previous study by Subakir et al, has shown that tissue from patients with menorrhagia may have elevated angiogenic potential.<sup>19,21</sup> Kooy et al in their study found that there was no significant difference in cellular proliferation in the stroma of endometrium from controls, patients with menorrhagia.<sup>19</sup> This indicated that the increase in endothelial cell proliferation in endometrium from patients with menorrhagia was specific and not the result of a general increase of cellular proliferation in the endometrium. Despite the increase of endothelial cell proliferation in endometrium from patients with menorrhagia, there was no change in endothelial cell concentration. A recent study by Wingfield et al revealed that endothelial cell proliferation was increased in the endometrium of patients with endometriosis compared with controls.<sup>22</sup> This increase was most marked during the proliferative phase of the menstrual cycle and was accompanied by an increase in the level of cellular proliferation in endometrial glands, stroma and surface epithelium.<sup>22</sup> Rogers have shown repeatedly that levels of endothelial cells proliferation within human endometrium

did not show any consistent pattern across the different stages of the menstrual cycle.<sup>5,19-22</sup>

Gargett et al found a highly significant correlation between the percentage of VEGF expressing vessels and vessels containing proliferating endothelial cells.<sup>23</sup> The percentage of proliferating vessels was higher in proliferative compared to secretory endometrium, but this was only statistically significant in the basalis layer. However, no statistical correlation was observed between VEGF production and endothelial cell proliferation in another study by Gargett et al.<sup>13</sup>

Mints et al observed that menorrhagia patients displayed statistically significantly more vessels/HPF in the secretory than in the proliferative phase measured in hot spots (P=0.011) and in random fields (P=0.007).<sup>24</sup> Studies concerning idiopathic menorrhagia suggested that the proliferative index of endothelial cells was increased in women with excess measured menstrual blood loss. Mints et al showed that endometrial blood vessels manifested an unusual morphology, characterized both by discontinuities (gaps) in endothelial immunostaining for CD34, VWF, and CD31, and by the localization of pericytes on the abluminal side of these gaps.<sup>25</sup> Moreover, these gaps were significantly larger in patients with idiopathic menorrhagia than in healthy subjects.

There was no consistency in the results obtained from the various studies attempting to correlate epithelial, stromal or total endometrial levels of VEGF mRNA and protein with phases of the menstrual cycle or endothelial cell proliferation.<sup>8,13</sup> A significantly greater percentage of focal VEGF-expressing microvessels were found during the proliferative phase as compared to the secretory phase, with the greatest numbers of immunopositive vessels within the subepithelial capillary plexus. The focal VEGF associated with microvessels was localised within marginating and adherent neutrophils.

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 to demonstrate ovulation.

## CONCLUSION

The conclusions drawn from present study were that the stromal and endothelial VEGF expression was comparatively higher in controls than menorrhagia patients. The endothelial proliferation was significantly higher in menorrhagia patients during late secretory phase of cycle than controls. In general, expression of both VEGF and PCNA was more in functional layer than basal layer in both cases as well as controls. On grading of PCNA positivity, it was observed that most cases and all controls in early proliferative phase did not express PCNA in blood vessels. The controls showed statistically significant in secretory phase for grading of PCNA

expression in blood vessels between cases and controls. We were able to demonstrate increased endothelial proliferation in patients in the premenstrual part of cycle. 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.

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