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VEGF: A Noble Immunohistochemical Marker in Diagnosis of Dysfunctional Uterine Bleeding?

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Abstract

Background: Dysfunctional uterine bleeding (DUB) is characterized by abnormal bleeding that is unrelated to any identifiable cause. The aim of this study is to establish that, DUB is a disorder of angiogenesis by detecting vascular endothelial growth factor (VEGF) expression on immunohistochemistry(IHC) study of DUB endometria. *Material and Methods*: It was a cross section study, undertaken at Index Medical College, Indore over a period of 18 months from March 2015 to July 2016. The study includes 100 endometrial dilation and curettage (D&C) and hysterectomy specimen of clinically diagnosed DUB patients. These specimens were studied for histopathology and expression of VEGF. *Results*: VEGF expression in endometria of DUB patients was significantly higher than in endometria of control group. There was also significant higher expression of VEGF in endometrial hyperplasia. *Conclusion*: Significantly over expression of VEGF in the endometrial glands in cases of DUB suggest that VEGF plays an important role in the pathogenesis of DUB

Keywords: DUB; Angiogenesis; VEGF; Endometrium.

Introduction

DUB is a common presentation in general gynaecological practice [1]. The definition of DUB that has been endorsed by the European Society for Human Reproduction and Embryology, is "excessive bleeding (excessive, heavy, prolonged or frequent) of uterine origin which is not due to demonstrable pelvic disease, complication of pregnancy or systemic disease".

The exact factors that lead to such abnormal bleeding are still unclear and unidentifiable; however, most women with DUB have an underlying aetiology of chronic anovulation with unopposed oestrogen stimulation of the endometrium [2]. Recent evidence has demonstrated that biochemical disturbances, including increased endometrial vascular fragility, disturbed endometrial angiogenesis, and inconsistency of the endothelial, epithelial, and stromal supporting structures in the local endometrial environment, may play an important role in the mechanism of DUB [3,4].

Angiogenesis is defined as the formation of new blood vessels from pre-existing vasculature. Vascularisation is essential for endometrial repair and growth, and the formation of new vessels depends on interactions between various hormones and growth factors [5,6]. Numerous endogenous and exogenous factors identified to have direct and indirect role in overall angiogenesis in the endometrium of DUB patients. Formation of blood vessels in human endometrium is controlled by hormones like oestrogen and progesterone [7]. However, mechanism of endometrial angiogenesis is a complex process. A large number of angiogenic factors and inhibitors have been identified in human endometrium beside hormones.

Although many growth factors can induce endometrial angiogenesis, VEGF family of proteins and their receptors play a crucial role as a potent stimulator of angiogenesis with many effects including endothelial cell proliferation, migration, and increase in vascular permeability and maintenance of vessel fragility [7]. VEGF correlates with physiological

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angiogenesis in the human endometrium and has a role in the development of the sub epithelial capillary plexus and functionalis microvessels during the proliferative phase of the menstrual cycle [8].

The hypothesis of the present study is that VEGF may be potential biomarkers of excessive angiogenesis in women with Dysfunctional uterine bleeding. It is proposed to study role of VEGF using immunohistochemical staining in endometrial tissues from women with DUB.

Materials and Methods

Study Group: This group consisted of 70 subjects who were clinically diagnosed as DUB patient. Endometrial biopsy or hysterectomy specimens of such patients were included.

Control Group: This group consists of 30 subjects who had undergone hysterectomy for causes not related to menorrhagia and also without any endometrial pathology e.g. Patients with prolapse and chronic cervicitis not responding to any medical treatment with normal menstrual cycle.

History and investigations of all the subjects was taken and following points were confirmed:

1. All subjects had proven fertility with no previous fertility problems and had delivered between one and six live births.
2. They had not received any hormonal medication or used any copper containing intrauterine device for the past 1 year.
3. Other potential variables that could influence menstrual bleeding such as diabetes mellitus, hypertension and thyroid function abnormalities were ruled out by performing specific tests.
4. Gestational history of all the patients had been taken and the pregnant subjects were excluded.
5. Patients with prolonged bleeding in only one isolated month were excluded.
6. Patients with fibroid, polyp, adenomyosis, endometrial carcinoma diagnosed histopathologically were excluded.
7. Following variables were recorded- Age, parity, history of bleeding, hemoglobin, Height, weight and endometrial thickness on USG.

Histopathology

- Histomorphological diagnosis was made along with endometrial dating.
- We made three histopathological sub-groups in our case group, first was proliferative phase, second group was simple hyperplasia and the last one was complex hyperplasia.
- The control group included normal patients in proliferative phase. This was done to avoid any kind of bias due to difference in histopathology. According to previous studies, the VEGF expression in the stroma is seen in Proliferative phase only.

Immunohistochemistry (IHC)

Standard IHC techniques were performed to demonstrate, quantify and localize the VEGF within the human endometrium.

Staining Pattern

Nuclear membrane, cytoplasm and cytoplasmic membrane.

Controls

Validation of all IHC protocols was carried out by the inclusion of control tissue slides. To assure the primary antibodies worked specifically to their target, the inclusion of positive and negative controls were required. For **negative controls**, the primary antibodies were omitted. For the **positive controls**, tissue samples, which are known to express the protein of interest i.e. human tonsil and kidney.

Inference

The expressions of VEGF were classified according to the following grading system as described by Tanaka et al [9]. Scores that correspond to the percentages of staining cells were defined as follows.

% Intensity of staining:

- 0 = negative response
- 1 = weak intensity
- 2 = moderate intensity
- 3 = strong intensity

% Percentage of positive cells:

- 0 = negative (0% immunopositive cells)
- 1 = positive immunoreaction in <25% of tumor cells cytoplasm
- 2 = positive immunoreaction in 26–50% of tumor cells cytoplasm
- 3 = positive immunoreaction in >50% of tumor cells cytoplasm

Final score was obtained by summing the two parameters, with the following interpretation for the immunohistochemical reaction:

No: A negative(-) immunoreaction for a score between 0 and 2

Low: A slightly positive(+) immunoreaction for a score between 3 and 4

High: A strongly positive(++) immunoreaction for a score between 5 and 6.

Results

The endometrial tissues from 70 women with DUB were taken and histopathological diagnosis was made. Three histopathological patterns were obtained. There were 33 cases with proliferative endometria, 29 with simple hyperplastic endometria, and 8 with complex hyperplastic endometria. The highest number of cases in the present study had proliferative phase endometrium.

Table 1 shows that out of 30 slides of control group, 50% show no VEGF expression in glands, while rest show low (Figure 1A) and high expression in 46.66% and 3.34% slides respectively. While in cases with clinically diagnosed DUB, 92.85% show low (Figure 1B) to high (Figure 1D) expression of VEGF in their endometrial glands with only 7.14% showing no glandular expression. But the VEGF expression in stroma was not significantly different. Out of 30 endometrium of control group, 33.33% subjects show stromal positivity (Figure 1A), and on studying cases, 42.85% show stromal positivity (Figure 1D).

Table 1: Table shows observed number and percentage of control and cases showing various grade of VEGF expression

Variables	Control (n=30)		Women with DUB (n=70)							
			Proliferative phase (n=33)		Simple Hyperplasia (n-29)		Complex Hyperplasia (n-8)		Total (n=70)	
	No.	%	No.	%	No.	%	No.	%	No.	%
Grade of VEGF expression in glands along with frequency and percentage										
No	15	50	4	12.12	1	3.44	0	0	5	7.14
Low	14	46.66	20	60.60	10	34.48	2	25	32	45.71
High	1	3.34	9	27.27	18	62.06	6	75	33	47.14
Low+High	15	50	29	87.87	28	96.55	8	100	65	92.85
Grade of VEGF expression in stroma along with frequency and percentage										
No	20	66.66	21	63.63	16	55.17	3	37.5	40	57.14
Low	10	33.33	11	33.33	11	37.93	4	50	26	37.14
High	0	0	1	3.03	2	6.89	1	12.5	4	5.714
Low+High	10	33.33	12	36.36	13	44.82	5	62.5	30	42.85

VEGF: Vascular endothelial growth factor, DUB: Dysfunctional Uterine Bleeding, No.: Number of cases, %: Percentage of cases

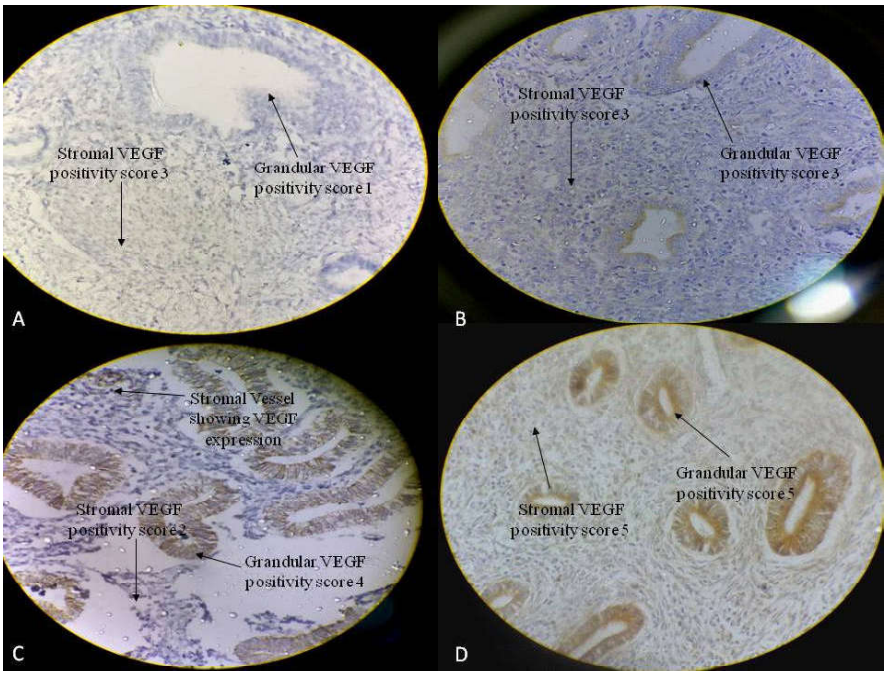

Fig. 1: (A) Microphotograph of normal endometria in proliferative phase showing very mild VEGF expression (40x); (B) Microphotograph of DUB endometria in proliferative phase showing low VEGF expression (40x); (C) Microphotograph of endometria of DUB patient in proliferative phase showing VEGF expression (40x); (D) Microphotograph of endometriaof DUB patient with simple hyperplasia showing extensive VEGF expression (40x)

The VEGF expression in endometrial glands is significantly higher in case group then in control group (p<.01). When we compared only first sub-group of cases (ie proliferative phase) with control group, it also comes out to be significantly increases (p<.05 value).

While on comparison of VEGF expression in stroma of control group and cases there was no statistical difference with P value >0.05.

When intra group comparison was done between the cases showing proliferative phase histology and cases showing hyperplastic endometrium, it also came out to be significant (p<.05). This result implies that VEGF expression in the glands depend upon the histological features. But this difference was not seen when intra group comparison of stromal expression of VEGF was done.

Discussion

Angiogenesis is a normal developmental and adult physiological process, requiring the coordinated action of a variety of growth factors and cell adhesion molecules in endothelial and mural cells. This study was designed to evaluate VEGF expression in DUB.

Greb RR et al show that the production and expression of VEGF in the case of normally menstruating primates is influenced by steroid hormone receptors of the endometrium [10].

Many workers have shown the increase in the activity and expression of VEGF in menorrhagic endometrium in comparison to normal endometrium [11,12]. Pilot information about the increased immunoreactivity in women with menorrhagia was suggested by S.K. Smith at al [13].

The intense VEGF expression has shown to stimulate the release of Nitric oxide and Prostaglandins from endothelial cells. This may further lead to endometrial vasodilation, increase in synthesis of tissue plasminogen and enhancement of vascular permeability [14,15].

In this study we have noted increased VEGF expression on IHC in endometrium of DUB patient when compared with control group. 65 out of 70 DUB patients (92.85%) showed VEGF positivity while only 15 out of 30 Subjects in control group (50%) had VEGF positivity in uterine endometrium. This result corresponds to various other studies that were conducted by comparing serum VEGF level of DUB patients with control group and also VEGF expression in endometrium of DUB patients and normal individuals.

Though there is a study by Shazia Malik on VEGF levels in menstrual blood, which shows decreased levels of VEGF in menorrhagia, for which they gave the explanation that the reduced concentration may just be a dilutional effect because of the increase rate of blood loss in menorrhagia patients [16].

Our study suggests that the predominant histological pattern in DUB is proliferative phase. 33 out of 70 patients fall in this pattern (47%). These cases on histopathological examination of endometrium showed endometrial glands with stratification, these glands are lined by tall columnar epithelium with tall nuclei and moderately dense stroma.

The other histopathological pattern was simple hyperplasia which accounts for 29 out of 70 cases (42%). On histopathological examination, these cases show increase gland to stroma ratio. Most of these glands were round and cystically dilated and show stratification.

The third histopathological pattern was complex hyperplasia which comprises of 8 out of 70 cases of DUB (11%). There endometrium on histopathological examination revealed crowding of glands with irregular shape and size. At places show back to back arrangement. Through these endometrium were not showing any signs of atypia.

The observations in study by Sadia Khan on different histopathological pattern of endometrium in DUB corresponds to our findings, which had maximum endometrium in proliferative phase followed by simple and complex hyperplasia [17].

Gheorman et al, in their study observed that, in normal endometrium the VEGF expression in glands is stable during the entire endometrial cycle which they postulated can be due to intensity modulation of IHC reaction to VEGF in various endometrial structure. Also it was noted by them that there is increase in VEGF positivity that correlates with the estradiol levels [18].

The VEGF expression in stroma was in focus by many authors however in our study the difference in VEGF expression in both groups was not significant though slight increase was observed in endometrium of DUB patients (Figure 1D). When compared with control group where VEGF was positive in 10 out of 30 individual (33.33%), case group shows stromal VEGF expression in 30 out of 70 individual (42.85%).

Regarding endometrial glandular cells, the reaction was more intensely positive and can be observed on more extended area than in similar structures of the normal endometrium, 65 out of 70 cases (92.85%) were

showing glandular VEGF positively in DUB endometrium in comparison to 15 out of 30 individuals (50%) of control group. High expression of VEGF is seen in 33 out of 70 (47.14%) DUB endometrium while is control group high expression is present in only 1 case (3.34%).

This increased expression of VEGF can be attributed to the steroid hormones as unopposed estrogen has been proven to be responsible for the increase VEGF in study by Greb et al. and in most of the cases of DUB, estrogen level is raised [19].

In our study there is significant difference between VEGF positivity of control group (50%) when compared with the positivity in proliferative phase DUB endometrium (Figure 1B) in which the VEGF expression was seen in 29 out of 33 cases (87.87%), which was contradictory to the Xinmei Zhang et all observations [20], This observation can be attributed to inclusion of disordered proliferative phase in the proliferative phase group of our study.

Also in this study VEGF expression in proliferative endometrium (Figure 1B) is significantly less than the hyperplastic endometrium (Figure 1C, Figure 1D). This finding suggests that the VEGF expression increases with the increase in endometrial complexity. Xinmei Zhang in their study also observed the same difference [20].

We have also seen in our study the correlation between VEGF expression endometrial thickness and histological pattern of the endometrium. This relation can be explained by the increase in VEGF expression in the hyperplastic endometrium in comparison to the proliferative endometrium.

Integrating the results of the previous studies with those obtained by the present study, it is implied that the increased VEGF expression may directly contribute to the occurrence of irregular uterine bleeding.

Conclusion

This is the 1st study of its kind in India, in which VEGF expression on Immunohistochemistry has been studied, taking in consideration different histological patterns. The prevalence of Dysfunctional uterine bleeding is increasing, so it is important to understand its exact pathology. Multiple factors are involved in the etiopathogenesis of DUB. Though the basic pathology being angiogenesis. The current study demonstrates the increase VEGF expression in the glands of DUB patients and also that the difference increases with hyperplasia.

The following relevant observations have been derived:-

- VEGF is important in the pathogenesis of DUB, its over expression in DUB endometrium proves its role.
- Over expression in hyperplastic stages clearly depits angiogenesis on IHC.

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