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Role of Serum HIF-1alpha and VEGF-A as an Angiogenic Factors in Women with Heavy Menstrual Bleeding

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Abstract

Aim: The purpose of this study to evaluate the levels of potent angiogenic factors, such as VEGF-A (Vascular Endothelial Growth Factor-A) and HIF-1 alpha (Hypoxia Induced Factor) in patients with Heavy Menstrual Bleeding (HMB). In addition, serum copper and ceruloplasmin as modulator of angiogenesis were investigated. We evaluated haemoglobin concentration and ET (Endometrial Thickness) as clinical and laboratory features of disease to investigate role of angiogenesis in etiology of HMB. **Methods/Analysis:** This case-control study was conducted on 120 females aged between 22-48 years with history of bleeding excessively for more than 3 months and 120 control samples. 5 ml of blood sample was collected from HMB patients and controls. Serum HIF-1 alpha, VEGF-A were measured by ELISA kit method. Copper was estimated using 3,5-dibromo-2-pyridylazo-N-ethyl-N-3 sulphopropyl aniline. Ceruloplasmin was estimated using P-Phenylenediamine (PPD) oxidase method. Endometrial thickness was obtained from Ultrasonography (USG). Hemoglobin was estimated using Drabkin's method. **Findings:** We observed higher concentration of VEGF-A as well as HIF-1 alpha in patient with HMB as compared to healthy women. The ROC (Receiver Operating Characteristic) curve was used to obtain optimal cut-off of VEGF-A as well as HIF-1 alpha to discriminate the HMB. **Conclusion:** Increase serum HIF-1alpha, VEGF-A and copper might be seen as predictors of HMB and increase efficacy of treatment.

Keywords: ET (Endometrial Thickness), HIF-1 Alpha (Hypoxia Induced Factor), HMB (Heavy Menstrual Bleeding), VEGF-A (Vascular Endothelial Growth Factor-A)

1. Introduction

Abnormal uterine bleeding refers to excessively heavy, prolonged or frequent bleeding which can lead to endometrial hyperplasia with growth of the endometrial vessel. Affected women usually have progressive severe anaemia. An Indian study reported 33.5% of patients in their OBG diagnosed with HMB[1]. The epidemiology of menstrual disorders was found to be 5-17 % in a WHO multicountry study among developing countries which included Indian women[2].

The recent FIGO classification of AUB (Abnormal Uterine Bleeding) is the PALM-COEIN classification. It refers to numerous gynaecological complications such as uterine tumours, hyperplasia and problem in blood clotting pathway, formation of polyp, adenomyosis, leiomyoma, ovulatory malfunction and bleeding of unknown origin and bleeding that is not classified. In

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general, all the segments of PALM can be diagnosed and visualized by imaging technique and histological analysis but COEIN category can not be analysed by aforementioned technique and consider to be non-structural[3].

Although medical treatment options are available, a significant proportion of women might need surgical removal of uterus and it has noticeable psychological and social impact on many women have led to its designation as a public health problem[4–7].

The female reproductive system is the principal place where angiogenesis occurs regularly in a physiological setting in the adult[8]. We hypothesis that the Copper-HIF-1α-VEGF-A pathway plays a significant role in endometrial angiogenesis. Dysregulation of this pathway could be the underlying cause of HMB. VEGF (Vascular Endothelial Growth Factor) appears to be one of the most important mediators of angiogenesis. Involvement of hypoxia, a key mediator of hypoxic condition (HIF-1α) is triggering point in the process of abnormal angiogenesis. Hypoxia-Inducible Factor 1 (HIF-1) is a transcription factor known to regulate the cellular response to hypoxia. The lack of oxygen (hypoxia) causes dimerization of two subunits (HIF-1α and HIF-1β) and initiate transcription of target genes, including factors involved in angiogenesis[9–11]. HIF has been identified in the human endometrium during the late secretory and menstrual phases[12]. It has been demonstrated that copper is involved in production and reinforcement of many number of angiogenic factors including VEGF-A[13,14]. Since copper is involved in hypoxic pathway (HIF-1alpha) that ultimately lead to synthesis of VEGF-A, hence it is considered to be important exogenous stimuli for angiogenesis. Copper and copper complexes have shown to be potential stimuli of angiogenesis in several animal models[15–17].

Although the micro-vessel density count is an established method of measuring angiogenesis[18], but measuring micro-vessel density is not suitable and applicable in every circumstances due to difficulties and infeasibility of this procedure, as a result evaluation of angiogenic biomarkers could be an informative to understand the extends of angiogenesis in HMB patients.

The hypothesis that increased VEGF-A expression during HMB when endometrial repair is initiated, and this increase is regulated by hypoxic conditions. Thus this study aimed at investigating the utility of VEGF-A, HIF-1 alpha, copper and ceruloplasmin as markers for differentiating HMB from the normal. Herein we demonstrated that circulating VEGF-A expression was 4-5 fold higher when it is compared with women with normal menstrual cycle.

2. Methods

2.1 Patients and Sampling

Ethical clearance from the Institutional Ethical committee (IEC 188/2013) was obtained to conduct this study. The patients and control population were non gravid women of reproductive age visiting the department of OBG, Kasturba hospital, Manipal. They were selected from the same geographical area to avoid any environmental factor and dietary factor that may have an effect on results. One hundred and twenty consecutive patients who underwent endometrial biopsy for HMB were recruited into the study. The control group (n = 120) were selected after intensive preliminary investigation among healthy women with regular menstrual cycle with no systemic disease visiting health check-up department, Kasturba Hospital, Manipal.

Patients with HMB were examined and graded according to FIGO classification system (PALM-COIN). Only patients who belonged to the endometrial (AUB-E) category were selected for the study. Mean Age for patients was 37.98 years (mean±SD, 37.98±7.07, range 22–48) and mean age for controls was 32.67 (mean±SD, 32.67 ± 8.01, range 19-45). All consenting women participated in the study.

The uterine cavity of patients with HMB appeared normal. Women with Inter Menstrual (IM) blood loss>80 mL per menstrual cycle selected for this study, this has been assessed according to pictorial blood loss assessment charts. Majority of Patients were in proliferative phase of menstrual cycle, as determined by the date of last menstrual period and biopsy reports. None of the study subjects smoked, or had used drugs or hormonal or intrauterine contraception for at least 3 months before biopsy, or had abnormal preoperative values for blood platelets, activated prothrombin, thromboplastin time, international normalized ratio or bleeding time. Currently the treatment in our hospital is prescribing medroxyprogesterone acetate or norethisterone acetate and in some cases surgical intervention (hysterectomy) required.

2.2 Exclusion Criteria

Women with symptoms of dysmenorrhea and dyspareurenia, and a history of endometriosis, were excluded from the study. Other potential variable that could effect menstrual bleeding such as high BMI, diabetes mellitus, hypertension and hypo and hyper thyroidism was ruled out. Those who were pregnant as well as women with other disorder such as fibroids, polyps and tumors were excluded from the study.

2.3 Endometrial Thickness

Ultrasonographic measurement of endometrial thickness was done in patients as well as controls.

2.4 Statistical Analysis

All data were analyzed by SPSS 15 (SPSS South Asia Bangalore). Normally distributed data are expressed as mean±SD and others as Median with IQR (Inter Quartile Range). Receiver Operating Characteristic (ROC) curves was used to determine the optimal cut-off values. The optimal cut-off points for each parameter measure were determined by the point of convergence of sensitivity and specificity.

3. Results

Mean Age of the patients participated in this study was 37.98 years and mean age for controls was 32.67 years. Means of VEGF-A, HIF-1α, Copper, Hb, endometrial thickness, Ceruloplasmin and age of patients and control group are presented in Table 2.

Since there were large variations in individual value of VEGF-A, HIF-1α and copper, hence it is expressed as median with IQR. The expression levels of serum HIF-1α and VEGF-A in HMB patients were expressed in the form of median with interquartile range 201.61 (124.92, 395.68) and 529.08 (391.71, 716.81) pg/ml respectively, serum level of HIF-1α and VEGF-A were significantly higher in patients when compared with control group 90.55 (59.60, 122.06) and 105.47 (78.53, 181.31) pg/ml. A statistically significant difference in median levels of serum HIF-1α and VEGF-A was observed in HMB patients as compared to the controls (P<0.0001).The serum copper as an exogenous compound is measured in the same patients 245.67 (188.35, 300.22) µg/dl and in controls 111.30 (96.67, 142.12) µg/dl and it is found to be statistically significant p<0.0001. Ultrasonographic measurement of endometrial thickness (mm) in both patients and controls performed and found to be significantly increased in patients as compared with controls and expressed in mean±SD (11.89±4.7, 5.19±1.50) respectively with p<0.0001. Due to excessive blood loss majority of patients were anemic and Hb value in patients and controls were (9.46±1.71 g/dl, 12.02±0.95 g/dl) respectively, with p<0.0001. Ceruloplasmin as a carrier of copper was highly elevated in patients as compared to the controls with mean±SD of (53.64±7.39 mg/dl, 32.14±7.80 mg/dl) respectively. The uncontrolled endometrial angiogenesis in HMB patients could be due to dominant hypoxic factor (HIF-1alpha) that may arise due to many exogenous factors like copper that may result in expression of VEGF-A, hence causing an increase in excessive endometrial blood vessels formation and endometrial growth.

Table 1. Demographical representation of patient's data

	Range	mean ± SD
Age	22-48	37.98 ± 7.07
H/O Bleeding	**Number of patients**	**Percentage**
<6 month	23	19.16%
>6 month	97	80.84%
Histopathology report		
Proliferative phase	105	87.5%
Secretory phase	15	12.5%
Nulliparous3	3	2.5%
Multiparous	117	97.5%
Live child birth		
0	3	2.5 %
1	13	10.83 %
2	67	55.83 %
3	32	26.66 %
4	5	4.18 %

Cut-off points of VEGF-A and copper, where sensitivity approximates specificity for this risk factor are depicted in Table 3. The values for VEGF-A is 257.2 pg/ml and copper has 141.17 µg/dl. This cut-off point for VEGF-A is of great importance since the value of 257.2 pg/ml has been reported as a significant higher value found in some other investigation. We speculate there are chains of critical factor involved in VEGF-A functional manifestation. High value for copper coincides with increase VEGF-A concentration. In this geographical area investigation have shown that exposure to copper have high incident mostly through drinking water that stored in copper vessel. A series of investigation were conducted in our

Table 2. Serum VEGF-A, HIF-1alpha and copper are expressed in median IQR and Hb, ET, Age and serum ceruloplasmin are expressed in mean±SD

Variable	Patients (n=120)	Control (n=120)	P value
VEGF-A(pg/ml)median IQR	529.08(78.53,181.31)	105.47(78.53, 181.31)	<0.0001
HIF-1alpha(pg/ml)median IQR	201.61(124.92,395.68)	90.55(59.60 , 122.06)	<0.0001
Copper(µg/dl) median IQR	245.64(188.35,300.22)	111.30(96.67, 142.12)	<0.0001
Hb(g/dl) (mean±SD)	9.46 ± 1.71	12.02 ± 0.95	<0.0001
ET(mm) (mean±SD)	11.89 ± 4.7	5.19 ± 1.50	<0.0001
Age (years) (mean ± SD)	37.98 ± 7.07	32.67 ± 8.01	<0.0001
Ceruloplasmin (mg/dl)	53.64±7.39	32.14 ± 7.80	<0.0001

Table 3. Angiogenic factors, Hb, ET, age and ceruloplasmin as predictors of HMB

Variable	cutoff	sensitivity	Specificity	Area under the curve
VEGF-A (pg/ml)	257.2	98%	93.5%	0.989
HIF-1alpha (pg/ml)	108.56	92%	64.5%	0.889
Copper (µg/dl)	141.17	88%	74.2%	0.924
Hb (g/dl)	11.35	82%	80.6%	0.915
ET (mm)	7.15	92%	87.1%	0.961
Age (years)	31.00	82%	48.4%	0.684
Ceruloplasmin (mg/dl)	40.6	96%	83.9%	0.971

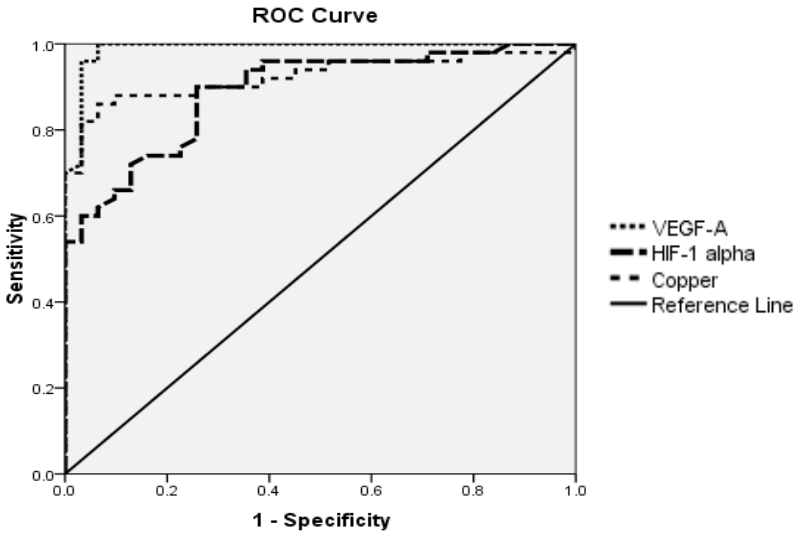

Figure 1. Comparison of three empirical ROC curves for VEGF-A, HIF-1alpha and copper.

laboratory since few years, now we believe cut-off point determination for VEGF-A and other angiogenic factors that involved in endometrial blood vessel formation must based on geographical area and race.

Based on the ROC curves for each of the biomarkers, the threshold cut-off values with highest sensitivity and specificity were identified and are depicted in Table 3. Cut-off point for HIF-1 alpha and Hb are 108.56 pg/ml , 11.35 g/dl respectively. HIF-1 alpha shows only 64.5% specificity and it not in close proximity of sensitivity. Hb has sensitivity approximates specificity. And cut-off point for Hb has found to be lower by approximately 0.5 g/dl than normal cut-off point for Hb value (12 g/ dl).

The calculated areas under the ROC curves for VEGF-A, HIF-1alpha and copper ranged 0.989, 0.889 and 0.924 respectively. The result indicate highest value obtained for area under the curve for all biomarkers were representative of sensitivity and specificity of test and probably could have more diagnostic value in patient with HMB. Three empirical ROC curves for VEGF, HIF-1 alpha and copper are represented in Figure 1.

The calculated areas under the ROC curves for Hb,

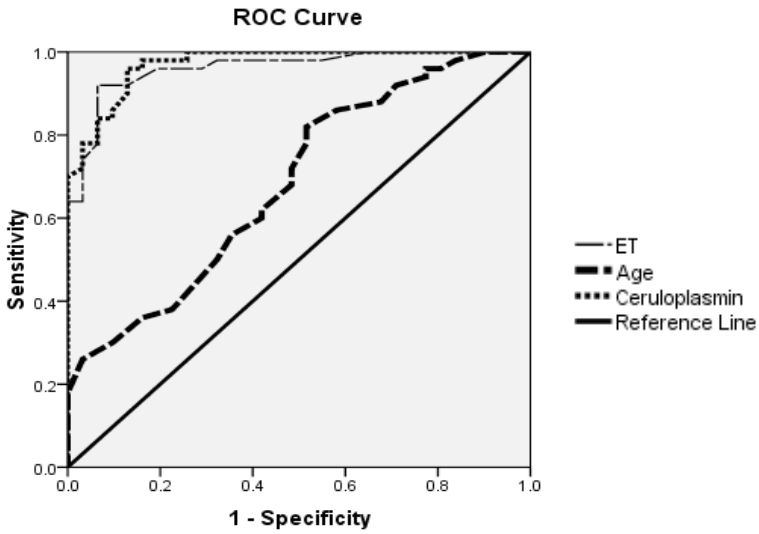

Figure 2. Comparison of three empirical ROC curves for ET (Endometrial Thickness), age and ceruloplasmin. Endometrial thicknesses as well as ceruloplasmin have higher sensitivity and specificity but age shows less sensitivity and specificity with respect to HMB.

ET, age and ceruloplasmin ranged 0.915, 0.961, 0.684 and 0.971 respectively. Cut-off point for Endometrial thickness is 7.15 (mm) that has a highest sensitivity and specificity and ceruloplasmin is 40.6 mg/dl that is well within the physiological range. Three empirical ROC curves for ET, age and ceruloplasmin are represented in Figure 2. Our data represent highest value obtained for area under the curve for all biomarkers but age shows lower value with this respect. For all parameters, the areas under the ROC curves most frequently were in highest category and decreases with age. This article is the first one that is reporting a cut-off point for ET and investigated angiogenic biomarkers but many other gynaecological conditions must be considered.

4. Discussion

Heavy menstrual bleeding is one of the important factors effecting women's quality of life and it is a main reason to seek gynaecological care for patient. A current available treatment option is hysterectomy for majority of patients with long standing heavy menstruation. Alternative medical options would lead to side effect such as unscheduled breakthrough bleeding. The ambiguity of heavy menstruation still remains unanswered. Enhancement of our knowledge of the mechanism of normal menstruation will provide an opportunity to identify novel targets for potential therapeutic development.

Progesterone withdrawal is the trigger for menstruation, and is associated with increased expression and generation of potent vaso-active prostaglandins. We described a VEGF-A involvement in endometrial angiogenesis in HMB patients and copper involvement of this process and signalling pathway associated with their regulation of HIF-1alpha, as potential novel therapeutic targets for menstrual bleeding disorders. The functional role of HIF-1alpha in human endometrium is unknown; it may be critical to the regeneration phase of the menstrual cycle. Tissue regeneration requires angiogenesis and matrix remodelling, processes clearly relevant to known HIF-1α target genes. We propose that abnormal angiogenesis is critical triggering phenomena due to hormonal imbalance and signalling initiate local events that lead to HIF-1alpha expression and downstream angiogenic gene expression (VEGF, CTGF, ET, Ang-2). This provides a coordinated mechanism for endometrial regeneration, that when dysregulated could result in heavy menstrual bleeding (menorrhagia).

There are several findings that relay copper as an enhancement of angiogenesis and numerous investigations that proves the involvement of copper in this process[19]. Exact role of copper remain to be elucidated.

This study shows that patients with heavy menstrual bleeding have highcirculating VEGF-A levels. Since VEGF-A is one of the most important soluble mediators of angiogenesis and its levels correlate with microvessel

density count, this finding confirms the increase of angiogenic activity in HMB.

In our study, VEGF-A and HIF-1alpha levels did not correlate with individual comparison among the patients with respect to clinical or laboratory features of the disease. Therefore, our observation does not clarify the expression level of VEGF-A and HIF-1alpha in the pathogenesis of HMB and the biological and clinical meaning of these results remains speculative. One speculation could be based on the fact that environmental factors like exposing to higher concentration of copper and hormonal involvement, progesterone and estrogen would result in uncontrolled shedding of endometrium and over long term stabilizing of angiogenic factors, VEGF-A and its receptor as well as HIF-1 alpha plays a role in pathogenesis of HMB. It is possible that the massive amount of circulating VEGF-A could be an independent factor for increase endometrial thickness in HMB patients.

The finding of increased angiogenic activity in HMB is consistent with the most recent literature demonstrating a role for angiogenesis, not only in the development of disease but also as a prognostic indicator. These observations may provide information for a novel treatment strategy for a disease in which the therapeutic armamentarium is quite poor. In this perspective, we have observed that VEGF-A and HIF-alpha measurement in the serum of HMB patients is an easy and reliable method and it can be used as a valuable tool for measuring the activity of angiogenesis.

We confirm that hypoxia increases endometrial VEGF-A production and describe, for the first time, its dependence upon endometrial HIF-1α during heavy menstruation. In addition, we reveal a novel copper mediated increase in endometrial VEGF expression that is independent of HIF-1α.

Previous immunehistochemical and in situ hybridization studies have also shown increased VEGF expression in the human endometrium during menstruation[20,21]. VEGF-A is a potent angiogenic factor that stimulates endothelial cell proliferation and migration[22].

Interestingly, a previous study of the human endometrium found significantly decreased VEGF mRNA and protein levels in women with HMB when compared with controls[23]. This finding is contradictory for our study that circulatory VEGF-A concentration increased up to 4-5 folds when compared with control. Therefore, delineation of the regulation of this important repair factor may provide novel therapeutic targets for HMB patients. PGs have previously been shown to increase VEGF expression in cancer cells[24,25], but we demonstrate a potential physiological role in women with probable pre malignant condition.

HIF-1 is a heterodimeric factor. The HIF-1α subunit undergoes proteasomal degradation in normoxia, whereas HIF-1β is constitutively expressed. In hypoxic conditions, HIF-1α binds with HIF-1β, translocates to the nucleus, and increases the transcription of target genes, including those involved in angiogenesis[24,26,27]. HIF-1α has been shown to be induced in late secretory and menstrual endometrium[28]. A hypoxic response element is present in the VEGF promoter region, and HIF-1 has been shown to regulate VEGF expression in hypoxic Hep3B cells[28]. In contrast, HIF-1-independent hypoxic regulation of VEGF has been described in various cancer cells[29]. Additional studies are required to determine whether a similar mechanism of action is present in normal endometrial tissue.

Our result indicates that:

- Women with heavy menstrual bleeding have elevated endometrial HIF-1alpha as well as VEGF-A expression compared to women with normal menstrual blood loss.
- Women with heavy menstrual bleeding have elevated serum copper level that can mimic VEGF-A.
- We believe that lower Hb level due to heavy blood loss further stabilize the hypoxic condition and lead to synthesis of HIF-1alpha.

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Disclosure summary: The authors have no conflicts of interest to declare.

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