Importance of Serum Copper and Vascular Endothelial Growth Factor (VEGF-A) Levels in Postmenopausal Bleeding

Afshan Rafi · Ramakrishna Devaki ·
K. Sabitha · Shruti Mohanty · Pragna Rao

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Abstract The main aim of this study is association of serum copper and vascular endothelial growth factor (VEGF-A) in postmenopausal bleeding (PMB) patients. Blood samples were collected from female patients suffering with postmenopausal bleeding ($n = 50$) as well as healthy females as controls ($n = 50$). Serum copper levels were estimated by spectrophotometric method and serum VEGF-A by ELISA technique and compared with ultrasonographic measurement of endometrial thickness in both patients and controls. A significant increase in serum copper levels and an almost twofold increase in serum VEGF-A was observed in DUB patients when compared with controls. Correlation (r) between serum VEGF-A levels and endometrial thickness was 0.96. Odds ratio for

copper, VEGF-A and combination of copper and VEGF-A was 0.0426, 0.0947 and 0.0313 respectively, in all these cases odds ratio was <1 . The abnormal angiogenesis in PMB could be due to increased serum copper levels, which in turn stimulates factors like VEGF-A, thereby causing an increase in endometrial growth.

Keywords Vascular endothelial growth factor · Postmenopausal bleeding · Serum copper · ELISA · VEGF-A

Introduction

Postmenopausal bleeding (PMB) is a common complaint in general gynecological practice. Women with PMB have around a 10 % chance of having endometrial carcinoma and therefore PMB always needs further evaluation [1]. Angiogenesis is the formation of new blood vessels from pre-existing vasculature and is a process fundamental to the human menstrual cycle. The uterine endometrium is a dynamic tissue that undergoes regular cycles of growth and breakdown, and has long been recognized as one of the few adult tissues where significant angiogenesis occurs on a routine, physiological basis. Abnormal angiogenesis may contribute to several different endometrial-related pathologies including endometrial cancer, endometriosis, menorrhagia and breakthrough bleeding [2].

Copper is an essential trace element for all the living organisms and is an obligatory cofactor of angiogenesis [3]. It stimulates endothelial cell migration and proliferation and activates vascular growth factors. Hence the angiogenic activity of beta-fibroblastic growth factor (β -FGF), vascular endothelial growth factor (VEGF), tumor necrosis factor-alpha (TNF- α) and interleukin-1 (IL-1)

Afshan Rafi, Devaki R, Shruti Mohanty and Pragna Rao contributed equally to this study.

A. Rafi · R. Devaki (✉) · S. Mohanty
Department of Biochemistry, Kamineni Institute of Medical Sciences, Sreepuram, Narkatpally, Nalgonda 508 254, Andhra Pradesh, India
e-mail: ramakrishna.d@kimsmedicalcollege.org

A. Rafi
e-mail: afshan_rafi2005@yahoo.com

S. Mohanty
e-mail: shruty.mohanty@gmail.com

K. Sabitha
Chalmeda Anandrao Institute of Medical Sciences, Bommakal, Karimnagar, Andhra Pradesh, India
e-mail: tejaswani19@gmail.com

P. Rao
Department of Biochemistry, Kasturba Medical College, Manipal University, Manipal 576104, Karnataka, India
e-mail: drpragnarao@gmail.com

were found to be copper dependent. Furthermore, copper repletion switches angiogenesis back “on” when a copper-sufficient diet is restored, providing evidence for a novel, physiologic, and metabolic control pathway of angiogenesis [4].

The 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 [5]. VEGF correlates with physiological angiogenesis in the human endometrium and has a role in the development of the sub epithelial capillary plexus and functional microvessels during the proliferative phase of the menstrual cycle [6]. The VEGF family currently comprises of the following members—VEGF-A, VEGF-B, VEGF-C, VEGF-D, and VEGF-E. VEGF-A, previously known as vascular permeability factor (VPF) is important in luteal angiogenesis. Both estradiol and progesterone induce the expression of VEGF-A [7]. Tumor growth and spread of tumor cells requires angiogenesis. Incipient angiogenesis is not induced by tumor cell hypoxia but probably by proangiogenic factors. During growth, tumors depend on a further induction of vascular development for adequate oxygen and nutrient supply. If the oxygen supply is insufficient, the resulting hypoxia stimulates angiogenesis through up regulation of VEGF [3]. Previous studies have shown that the angiogenic activity of the growth factors (VEGF, β -FGF) were copper dependent and copper reduction resulted in the inhibition of their angiogenic activity [4].

The hypothesis of the present study is that serum levels of copper and VEGF-A may be potential biomarkers of excessive angiogenesis in women with postmenopausal bleeding.

Materials and Methods

Patient Recruitment

This study was conducted at a rural hospital in south India. The patients as well as the control population were drawn from the same geographical area in order to ensure that the same environmental conditions and dietary habits were prevalent for both the groups. Here, the awareness of contraceptive measures for birth control is extremely poor. Fifty consecutive patients who had postmenopausal bleeding were recruited into the study. All patients agreed to participate in the study. The recruitment process consisted of taking a thorough history with a well designed proforma. History of environmental or occupational expo-

sure to copper was taken as was the use of copper vessels for cooking and storing drinking water.

Patients were mostly in the proliferative phase of the menstrual cycle, as determined by the date of the last menstrual period and biopsy report. The women were between the ages of 50–72 years (mean age 58 years). Only patients who had irregular and excessive uterine bleeding continuously for more than 3 months were included in the study. Patients with prolonged bleeding in only one isolated month were not included as having dysfunctional uterine bleeding. They had no other associated gynecological diseases. All patients earlier had regular (27–30 days) menstrual cycles, lasting for 3–6 days and no history of dysmenorrhea during menstruation. All patients had proven fertility with no previous fertility problems and had delivered between one and six live infants with the last delivery being 1–3 years prior to the history of postmenopausal bleeding. They had not received any hormonal medication or used any copper containing intrauterine device for the past 1 year. None of these women were smokers. Other potential variables that could influence menstrual bleeding such as high body mass index (BMI), diabetes mellitus, hypertension and hypo- and hyperthyroidism was ruled out. Women who were pregnant or who had bleeding disorders were excluded from the study as were women with other diseases like fibroids, polyps and tumors. Height, weight and BMI were recorded.

The control population consisted 50 healthy age matched non-pregnant women who came for a dental check up to the hospital with a history of normal menstrual cycles. They had no history of any hormonal medication or use of any copper containing intrauterine device for the past 1 year. None of these women were smokers or had a history of diabetes mellitus, hypertension or thyroid disorders. The study was approved by the Institutional Review Board. The patient's consent for the study was obtained.

A random venous blood sample was collected for estimating serum copper and serum VEGF-A levels. Serum copper levels were estimated by using 3,5-di bromo pyridazole sulphanilic acid as coloring agent. Serum VEGF-A levels were estimated by sandwich ELISA technique. Quality controls were assessed in every series of samples to check the reproducibility and accuracy of the measurements.

Statistical Analysis

The statistical analysis was performed using SPSS software 11.0 version. Data was expressed as mean $\pm$ SD. Significance of difference between the patient and control groups observed is assessed by using the unpaired Student *t* test. *p* value <0.05 was considered to be significant.

Table 1 Demographic details and the levels of serum copper and VEGF-A of the postmenopausal bleeding (PMB) patient and controls

	Controls (<i>n</i> = 50) Range	Mean ± SD	PMB patients (<i>n</i> = 50) Range	Mean ± SD
Age (years)	48–75	58.56 ± 7.21	50–72	58.52 ± 6.09
Parity	0–10	4.04 ± 1.95	0–12	3.8 ± 2.24
BMI	19.05–22.22	19.83 ± 0.82	15.56–25.32	20.55 ± 2.35*
History of bleeding (in days)	3–5	4.14 ± 0.95	10–21	13.9 ± 4*
Ultrasonographic measured endometrial thickness (mm)	1–2	1.64 ± 0.49	4–11	7.64 ± 2.23*
Hemoglobin (gm %)	9.6–13.2	10.84 ± 0.88	7.1–12.2	10.08 ± 1.10**
Serum copper (µg/dl)	84–114	93.68 ± 8.32	102–214	162.92 ± 34.88*
Serum VEGF-A (pg/ml)	125–225	173 ± 38.13	225–950	387 ± 180.73*

BMI body mass index

* *p* value <0.0001, ** <0.01

Results

To see the effect of copper induced VEGF-A expression in postmenopausal bleeding, we have studied 50 PMB patients aged between 48 and 75 years (Group I) against 50 age matched non-pregnant healthy women with normal menstrual cycle (Group II). Among the demographic details of the patients and control when compared, especially serum copper, VEGF-A, and BMI levels were significantly increased ($p < 0.0001$) in PMB patients (Table 1). The majority of PMB patients (58 %) were between 48 and 75 years. The mean of parity among patient and control group was not significantly different between the two groups. The ultrasonographically measured endometrial thickness showed almost threefold increase thickness among the patient group ($p < 0.0001$).

Serum copper, which is an obligatory cofactor of angiogenesis was measured in patients and controls. Serum copper levels were higher (162.92 ± 34.88 µg/ml) in PMB patients than in controls (93.68 ± 8.32 µg/ml) (Table 1). It showed a twofold increase in patients when compared with controls and was highly significant ($p < 0.0001$).

Copper and VEGF-A are sensitive indicators of PMB (Table 2), the specificity increases when both the parameters are measured together with a high positive likelihood ratio. Analysis of the data produced the 2×2 table shown in Table 2. Odds ratio for copper, VEGF-A and combination of copper and VEGF-A was 0.0426, 0.0947 and 0.0313 respectively, in all these cases odds ratio was <1. It indicates that copper induces the VEGF-A expression which in turn increases the angiogenesis in endometrium leads to PMB. Serum copper levels alone did not correlate with endometrial thickness.

A significant positive correlation between serum copper and serum VEGF-A levels was observed ($r = 0.339$, $p < 0.01$). The present study also showed a significant negative correlation ($r = -0.96$) between serum VEGF-A

Table 2 Sensitivity and specificity of serum copper and VEGF-A testing in patients with menorrhagia

	Copper	VEGF-A	Combination of copper and VEGF-A
Sensitivity	0.94	0.90	0.90
Specificity	0.60	0.54	0.78
Positive likelihood ratio	2.35	1.96	4.09
Negative likelihood ratio	0.10	0.19	0.13
Odd's ratio	0.0426	0.0947	0.0313
95 % confidence intervals	0.0116–0.1557	0.0322–0.2783	0.01–0.0981

Table 3 Correlation between serum copper, serum VEGF-A and endometrial thickness in postmenopausal bleeding patients

Parameters	Serum copper (µg/dl)	Serum VEGF-A (pg/ml)	Endometrial thickness (mm)
Serum copper (µg/dl)	1	$r = 0.339$ $p = 0.0011$	$r = -0.038$ $p = 0.639$
Serum VEGF-A (pg/ml)	$r = 0.339$ $p = 0.0012$	1	$r = -0.96$ $p = 0.011$
Endometrial thickness (mm)	$r = -0.038$ $p = 0.839$	$r = -0.94$ $p = 0.017$	1

and endometrial thickness (Table 3). VEGF-A expression occur in the upper zone stroma of human endometrium. During menstruation only the deeper zones persist after the upper zones have sloughed away (11). Additionally, after menstrual sloughing of the upper zones, VEGF-A and its receptors are coordinately expressed with intense expression of VEGF-A in the newly formed surface epithelium and significant increase in both VEGFR1 and VEGFR2

Table 4 Comparison of serum copper and serum VEGF-A levels in postmenopausal bleeding patients due to benign and malignant conditions

	Benign lesions (<i>n</i> = 17)	Malignant lesions (<i>n</i> = 8)	<i>p</i> value
Serum copper ($\mu\text{g/dl}$)	145.24 $\pm$ 27.13	200.50 $\pm$ 10.80	<0.0001
Serum VEGF-A (pg/ml)	295.59 $\pm$ 61.39	581.25 $\pm$ 199.89	<0.0001

expression in multiple profiles of small vessels subjacent to the surface epithelium. In PMB patients Hb level are significantly decreased (10.08 ± 1.10 , $p < 0.01$) when compared to controls (10.84 ± 0.88). This may be due to excessive bleeding in PMB patients.

In this present study comparative study was done in patients with postmenopausal bleeding due to benign and malignant conditions by estimating VEGF and serum copper. Studies showed that significant increase in serum copper ($p < 0.0001$) and a twofold increase in serum VEGF-A ($p < 0.0001$) levels in patients with postmenopausal bleeding due to malignancy when compared with patients with bleeding due to benign conditions like cervicitis and fibroids (Table 4).

Discussion

Angiogenesis is the formation of new blood vessels from pre-existing vasculature and is a process fundamental to the human menstrual cycle. The uterine endometrium is a dynamic tissue that undergoes regular cycles of growth and breakdown, where significant angiogenesis occurs on a routine, physiological basis. Abnormal angiogenesis may contribute to several different endometrial-related pathologies including endometrial cancer, endometriosis, menorrhagia and breakthrough bleeding [2].

In a previous study [7], the role of VEGF and copper was reported in dysfunctional uterine bleeding patients, whereas in this paper we are reporting our results in connection with the same parameters in PMB patients.

Copper is an obligatory cofactor of angiogenesis. It stimulates endothelial cell migration and proliferation and activates vascular growth factors. Hence the angiogenic activity of beta-fibroblastic growth factor (β -FGF), vascular endothelial growth factor (VEGF), tumor necrosis factor-alpha (TNF- α) and interleukin-1 (IL-1) were found to be copper dependent [5].

Copper has been shown to influence the bioactivity or production of a number of angiogenic factors including VEGF-A [5, 8] and shares some of the pathways utilized by hypoxia to regulate VEGF-A expression [2, 5] demonstrated

that copper is having anti-tumor activity [9]. Copper is also required in hemoglobin synthesis and in the catalysis of metabolic oxidation [10]. A specific amount of copper appears to be vital for angiogenesis to occur [11, 12]. Copper or copper complexes have been shown to directly stimulate angiogenesis in several animal model systems while copper chelation has been shown to inhibit angiogenesis [13]. Copper containing intra uterine devices (IUD) increases inflammatory action and uterine bleeding [14].

Initial studies have been done on serum, secretory endometrium and menstrual blood copper levels in women using copper-T IUD. There was no change in the serum copper levels, but the secretory endometrium and menstrual blood copper levels were significantly increased in the endometrial tissue samples which were taken after insertion of IUD when compared with samples taken before insertion. With these results it was concluded that copper is not stored in basal layers of the endometrium but is continually released from the copper IUDs [15]. Thus previously serum copper levels were studied only in women using copper-T IUD.

Vaginal bleeding is the presenting sign in more than 90 % of postmenopausal patients with endometrial carcinoma. However, depending on age and risk factors, 1–14 % will have endometrial cancer [16, 17]. Tsikouras et al. [18] showed retrospective study, which included 275 patients with PMB, reported a 8.16 % prevalence of endometrial cancer in women with PMB and endometrial thickness <4 mm. Using a maximum 5-mm endometrial thickness, in a study of 182 women with PMB [19]. According to the results of Doorn et al. [20] study, 8 % of the women with recurrent bleeding turned out to have an endometrial carcinoma. These women had presented with a first episode of PMB.

In the present study serum copper and VEGF-A levels were studied in two groups of women, one with PMB and other with their respective age matched healthy controls.

In the present study comparative study of patients with postmenopausal bleeding due to benign and malignant conditions by estimating VEGF and serum copper, showed that significant increase in serum copper and a twofold increase in serum VEGF-A levels in patients with postmenopausal bleeding due to malignancy when compared with patients with bleeding due to benign conditions like cervicitis and fibroids. This suggests that serum copper and VEGF-A levels are higher in malignancies when compared with benign lesions.

From a clinical perspective, in management of postmenopausal bleeding haematinics are usually given to treat anemia due to postmenopausal bleeding [21]. Most of these haematinics contain copper in them as copper helps to increase the absorption of iron [22]. This could lead to further elevation of copper levels which further stimulates

VEGF-A leading to angiogenesis, resulting in postmenopausal bleeding or increased angiogenesis.

Conflict of interest The authors declare that they have no competing interests.

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