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Serum Copper and Vascular Endothelial Growth Factor (VEGF-A) in Dysfunctional Uterine Bleeding

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ABSTRACT

Over the past 20 years there has been an upsurge of interest proper cause of abnormal angiogenesis of DUB. This study particularly focused on the association of serum copper and vascular endothelial growth factor (VEGF-A) in patients with Dysfunctional Uterine Bleeding (DUB). Blood samples were collected from female patients suffering with DUB (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 two fold 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 0.97. Odd 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 less than one. The abnormal angiogenesis in DUB could be due to increased serum copper levels, which in turn stimulates factors like VEGF-A, thereby causing an increase in endometrial growth.

Key words: Vascular endothelial growth factor, dysfunctional uterine bleeding, serum copper, ELISA, endometrium, odds ratio, angiogenesis

INTRODUCTION

Human endometrium expresses many angiogenic mediators and their specific receptors (Girling and Rogers, 2005; Smith, 2001), including many growth factors, cytokines and proteases (Achache and Revel, 2006). Any alterations in the expression of these mediators may lead to inappropriate tissue regeneration, embryo implantation failure, dysfunctional uterine bleeding, endometriosis, and endometrial cancer (Ferenczy, 2003).

A prototypical angiogenic factor, vascular endothelial growth factor, (VEGF-A), is a key mediator of neovascularization (Baiomy *et al*., 2004). The 43-kD, dimeric, heparin-binding glycoprotein is specifically mitogenic for endothelial cells (Folkman and Klagsbrun, 1987). The dominant VEGF-A mRNA transcripts expressed by human endometrium and primary human endometrial cells encode the VEGF 165 (Ferrara *et al*., 1992) and VEGF 121 (Torry *et al*., 1996)

proteins. Previous studies have demonstrated that estrogen increases the secretion of VEGF in uterine cells and is a central regulator of the uterine vasculature (Cullinan-Bove and Koos, 1993; Herve *et al*., 2006).

Copper has been shown to influence the bioactivity or production of a number of angiogenic factors including VEGF-A (Harris, 2004; Sen *et al*., 2002) and shares some of the pathways utilized by hypoxia to regulate VEGF-A expression (Sen *et al*., 2002). Jun *et al*. (2007) demonstrated that copper is having anti tumor activity (Jun *et al*., 2007). Copper is also required in hemoglobin synthesis and in the catalysis of metabolic oxidation (Al-Numair, 2006). A specific amount of copper appears to be vital for angiogenesis to occur (Pan *et al*., 2002; Yoshii *et al*., 2001). 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 (Finney *et al*., 2007). Copper containing Intra Uterine Devices (IUD) increases inflammatory action and uterine bleeding (Kulier *et al*., 2006).

It is hypothesized that elevated serum copper and VEGF-A levels could be associated with menorrhagia, which is excessively heavy menstrual bleeding in the absence of a well defined pelvic pathology, often referred to as Dysfunctional Uterine Bleeding (DUB). Whether serum copper and VEGF-A levels correlated with ultrasound studies on endometrial thickness in DUB was also studied. The current study is a preliminary one to explore the possibility of using serum copper and VEGF-A levels as non invasive biomarkers of DUB.

MATERIALS AND METHODS

This research project was conducted from 18th September 2008 to 7th October 2010.

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 undergoing endometrial biopsy for DUB were recruited into the study. All patients agreed to participate in the study. The recruitment process consisted of taking history with a well designed proforma. History of environmental or occupational exposure 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 18-45 years (mean age 30 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 DUB. 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, Diabetes Mellitus, hypertension and hypo and hyper thyroidism 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. Ultrasonographic measurement of endometrial thickness was done in patients with DUB as well as in the control group.

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±SD. Significance of difference between the patient and control groups observed was assessed by using the unpaired student 't' test. A p-value less than 0.05 was considered to be significant. The odds ratio for 95% confidence limits was calculated using a 2×2 table (Simple Interactive Statistical Analysis at http://www.quantitativeskills.com/sisa/statistics/two2hlp.htm).

RESULTS

To see the effect of copper induced VEGF-A expression in dysfunctional uterine bleeding, we have studied 50 DUB patients aged between 15 to 45 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 DUB patients (Table 1). The majority of DUB patients (48%) were between 26-35 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 3 fold 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 (315±156.82 pg mL^{-1}) in DUB patients than in controls

Table 1: Demographic details and the levels of serum copper and VEGF-A of the DUB patients and controls

	Controls (n = 50)		DUB patients (n = 50)	
Parameters	Range	Mean+SD	Range	Mean±SD
Age (years)	18-45	29.9±7.8	17-45	30.44±7.75
Parity	0-5		0-6	
BMI (body mass index)	17.3-22.22	20.02±1.21	15.11-29.44	22.48±3.01*
History of bleeding (in days)	3-5	4.14±0.95	10-21	13.9±4*
Ultrasonographic measured endometrial thickness (mm)	1-4	2.54±0.81	6-14	7.54±1.45*
Hemoglobin (gm %)	9.6-12.5	10.63±0.66	6.5-10.4	8.10±1.07*
Serum Copper (μg dL^{-1})	82-134	104.08±11.43	83-206	155.94±0.92*
Serum VEGF-A (pg mL^{-1})	100-300	181.5±59.72	150-900	315±156.82

*$p<0.0001$, **$p<0.05$

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

Parameters	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

*p<0.01. Sensitivity near 1 is highly significant and states that serum copper and serum VEGF-A will be high in women with menorrhagia

Table 3: Correlation between serum Copper, serum VEGF- A and endometrial thickness in DUB patients

Parameters	Serum copper (μg dL^{-1})	Serum VEGF-A (pg mL^{-1})	Endometrial thickness (mm)
Serum copper (μg dL^{-1})	1	r = 0.439	r = -0.048
		p = 0.0014	p = 0.739
Serum VEGF-A (pg mL^{-1})	r = 0.439	1	r = -0.97
	p = 0.0014		p = 0.019
Endometrial thickness (mm)	r = -0.048	r = -0.97	1
	p = 0.739	p = 0.019	

r = Correlation, p = p value

(181.5±59.72 pg mL^{-1}) (Table 1). It showed a two fold increase in patients when compared with controls and was highly significant (p<0.0001).

Copper and VEGF-A are sensitive indicators of DUB (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. Odd 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 less than 1. It indicates that copper induces the VEGF-A expression which in turn increases the angiogenesis in endometrium leading to DUB. 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 = 0.439, p<0.01). The present study also showed a significant negative correlation (r = -0.97) between serum VEGF-A 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 (Martin *et al*., 2003). 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 expression in multiple profiles of small vessels subjacent to the surface epithelium.

In DUB patients Hb level were significantly decreased (8.10±1.07, p<0.0001) when compared to control (10.63±0.66). This may be due to excessive bleeding in DUB patients.

DISCUSSION

Angiogenesis is a fundamental developmental and adult physiological process, requiring the coordinated action of a variety of growth factors and cell-adhesion molecules in endothelial and mural cells (Mocellin, 2006). This study was designed to evaluate the copper inducing properties

of VEGF-A expression leading to increase angiogenesis, which echoed the well-established mechanism responsible for the angiogenic properties of copper. Whereas a direct role of copper to facilitate angiogenesis has been evident since two decades ago (Sen *et al*., 2002) the specific targets of copper action remained unclear.

The environment has several sources of copper. Excessive copper is often involved in cancer and may be a risk factor in estrogen-dependent cancers. Research has shown that there is a 72% increase in the copper content of malignant tumors of the ovary, uterus and cervix (Margalioth *et al*., 1983). In humans, exposure to metals occurs primarily through dietary sources of food and water (Krizek *et al*., 1997; Peraza *et al*., 1998) air, cigarette smoke (Krizek *et al*., 1997) and occupational exposure (Krizek *et al*., 1997; Peraza *et al*., 1998) and can lead to significant accumulation in the body. Although the precise roles of metals and metalloids in breast cancer and other endocrine-related diseases remain to be determined, their ability to function as potent estrogens suggests that they may be an important class of endocrine disrupters. Recent studies suggest that metals may represent a new class of endocrine disrupters (Garcia-Morales *et al*., 1994; Stoica *et al*., 2000). The ability of metals to bind with high affinity and activate estrogen receptors suggests that, at environmentally relevant doses these compounds may pose a risk for endocrine-related diseases. In fact, exposure to metals is associated with endocrine imbalances and significant reproductive toxicity (Gerber *et al*., 1980; Uzych, 1985).

Copper is also associated with the actions of estrogens. Copper accumulates in normal and neoplastic estrogen target tissues, such as uterus and mammary gland (Fuchs *et al*., 1986; Schwartz *et al*., 1974) and appears to modulate the sensitivity of these tissues to both estrogens and antiestrogens. The ability of metals to activate Estrogen Receptor (ER) was measured in the human breast cancer cell line, MCF-7. The ability of the metals to activate a chimeric receptor containing the hormone-binding domain of ER suggests that their effects are mediated through the hormone binding domain (Martin *et al*., 2003). Copper is an obligatory cofactor of angiogenesis. It stimulates endothelial cell migration and proliferation and activates vascular growth factors. Increased levels of copper induce the serious toxic implications such as nausea, vomiting, hemolysis, methemoglobinemia, hepatorenal failure, chronic tubulo-interstitial nephritis, metabolic acidosis, septicemia, shock, carcinogenic effects and death in human beings (Almansour, 2006).

In the present study, serum copper and VEGF-A levels were studied in two groups of women, one with DUB with their respective age matched healthy controls as potential biomarkers of excessive angiogenesis. Leone *et al*. (2006) showed that, the copper effect is mediated at least in part by the VEGF signalling pathway. The present study also showed that copper induces the VEGF-A levels leads to angiogenesis in endometrium. 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. Interestingly, VEGFR2 expression is also dramatically increased in both the human and macaque endometrium in the same upper zone stroma of premenstrual/menstrual phase that strongly expresses VEGF-A. This coordinated expression of VEGF-VEGFR2 in the upper zone stroma has been suggested to be involved in the stimulation of matrix metalloproteinases (MMPs) production. 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 expression in multiple profiles of small vessels subjacent to the surface epithelium. This may explain the observed significant negative correlation between VEGF-A levels and endometrial thickness. From a clinical perspective, in management of DUB haematinics are usually given to treat anaemia due to

excessive bleeding. Most of these haematinics contain copper in them as copper helps to increases the absorption of iron. This could lead to further elevation of copper levels which further stimulates VEGF-A leading to angiogenesis, thereby resulting in more heavy bleeding. According to the study done by Harris ED, the copper effect is mediated at least in part by the VEGF signalling pathway (Leone *et al*., 2006).

CONCLUSION

In conclusion, we describe the effects of elevated serum copper leading to an increase in uterine angiogenesis in patients with menorrhagia which may be secondary to an E2 up-regulation of VEGF expression.

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