REVIEW ARTICLE

Role of Copper and Vascular Endothelial Growth Factor (VEGF) on Endometrial Angiogenesis

*Yousef Rezaei Chianeh[1], Pragna Rao[1]**

[1]*Department of Biochemistry, Kasturba Medical College, Manipal University, Manipal -576104 (Karnataka), India*

Abstract:

The formation of new blood vessels is the initial step in neovascularisation. The first stage in angiogenesis is the activation of endothelial cells. Copper ions stimulate proliferation and immigration of endothelial cells. It has been shown that serum copper concentration increases as the cancer disease progresses and correlates with tumour incidence and burden. Copper ions also activate several proangiogenic factors, e.g., vascular endothelial growth factor, basic fibroblast growth factor, and interleukin 1. This review concerns a brief introduction into the basics of blood vessel development as well as the regulatory mechanisms of this process. The role of copper ions in angiogenesis is discussed.

Key words: Angiogenesis, Vascular Endothelial Growth Factor (VEGF), Copper, Ceruloplasmin.

Introduction:

Angiogenesis, the formation of new capillaries from existing vasculature, is a critical process in normal physiology as well as several physiopathologies. Judah Folkman recognized this fact in a 1971 hypothesis that stated "tumour growth requires an increment in capillary growth [1]. Variable angiogenic factors that comprise the network of components that respond to tumour cell stimuli were identified and, in time, a sequence of cellular events was hypothesized. Such events include the activation of factors that mediate migration, mitosis, and differentiation of endothelial cells, and the reshaping of matrix proteins into the familiar tubular structure of capillary anatomy, each of which has a rather specific requirement for copper. Copper is an essential trace element for all the living organisms [2] and is one of the key requirements for angiogenesis. Hence the activity of several angiogenic factors directly or indirectly depends upon it [3]. The intricate steps of angiogenesis are a multifactorial process that involves participation of many growth factors. The role of copper containing enzymes, ceruloplasmin (ferroxidase I) and ferroxidase II that have the capacity to oxidize ferrous iron (Fe2+) to ferric iron (Fe3+), the form of iron that can be loaded onto the protein transferrin for transport to the site of red blood cell formation and they act as an instigators of angiogenesis. Although the ferroxidase activity of these two cuproenzymes has not yet been proven to be physiologically significant, the fact that iron mobilization from storage sites is impaired in copper deficiency supports their role in iron metabolism [4, 5]. In this review article we emphasize the role of copper and copper containing compound in physiopathology of angiogenesis in Dysfunctional Uterine Bleeding (DUB).

Dysfunctional Uterine Bleeding (DUB) refers as an excessively heavy, prolonged or frequent

bleeding (menorrhagia) which is not caused by pregnancy or any recognizable pelvic or systemic disease [6]. The etiology of DUB could be hormonal imbalance, which can lead to endometrial hyperplasia [7] and occurs from the endometrial capillaries and smaller vessels [8]. There are several factors involved in the regulation of endothelial cell growth, function and angiogenesis in DUB. The most studied are Vascular Endothelial Growth Factor (VEGF) [9, 10] and endothelial nitric oxide synthase (eNOS) [11, 12]. VEGF has been shown to play a pivotal role in the initiation and formation of blood vessels, which involves endothelial cell growth and differentiation [9, 10]. Vascular endothelial growth factor (VEGF) is a signal protein produced by cells that stimulates vasculogenesis and angiogenesis. It is part of the system that restores the oxygen supply to tissues when blood circulation is inadequate. It is been demonstrated that copper is required for VEGF expression through its regulation of Hypoxia-Inducible Factor (HIF) activity. Hypoxia-inducible factors (HIFs) are transcription factors that respond to changes in available oxygen in the cellular environment, specifically, to decreases in oxygen, or hypoxia. However, the mechanisms underlying these two actions are different. It has been known that hypoxia-inducible factor 1-alpha, also known as HIF-1α, is a protein that in humans is encoded by the HIF1A gene which is the critical transcription factor for VEGF expression. The activity of HIF-1α is mainly regulated by its intracellular stability and higher concentration; hence concentration of HIF-1á at lower level may not induce angiogenesis [13, 14]. Metals such as cobalt [15, 16, 17] nickel [15, 17] and copper [18, 15] can inhibit the process of HIF-1α degradation, and promote stabilization thus leading to its accumulation and activation. Therefore, cells exposed to high levels of cobalt, nickel or copper leads to overproduction of VEGF [18, 15, 16, 17]. It is been shown that activation of HIF-1α transcription activity requires copper. Copper depletion results in suppression of HIF-1α activation as well as inhibition of VEGF expression [19, 20]. Most studies have used high levels of copper to examine its effect on VEGF production and cell growth, [21] leading to a conclusion that copper stimulation of cell growth is mediated by VEGF production. However, one study has clearly shown a distinction between stimulation of cell growth by physiologically relevant levels of copper and that by copper overload. Under the experimental condition, copper did not increase VEGF production, but copper was clearly required for VEGF expression because copper chelation by tetraethylenepentamine (TEPA) inhibited VEGF expression. Corresponding to this effect was the inhibition by TEPA of normal cell growth, an effect that could be reversed by excess copper. Therefore, under physiological condition, copper is required for VEGF expression to maintain normal cell growth [22]. It is important to note that both genes for eNOS and VEGF are under the regulation of HIF-1α, copper is required for HIF-1α transcriptional activation and high levels of copper induce HIF-1α accumulation in the cell [18, 15]. Therefore, this would suggest that the increase in eNOS transcription results from the effect of copper on mechanisms other than the regulation under HIF-1α.

Copper and Neovascularization:

Copper has been shown to influence the bioactivity or production of a number of angiogenic factors [23, 24] and shares some of the pathways utilized by hypoxia to regulate VEGF-A expression [25, 23]. Vascular endothelial growth factor A (VEGF-A) is a protein that in humans is encoded by the VEGF-A gene. The outgrowth of blood vessels streaming from the implant was interpreted to result from the migration of a specific subset of endothelial cells in response to an unknown angiogenic stimulus. Copper was thought at first to be acting as a chemotaxic agent [26]. Later, it is been shown by, Hannan and MacAuslan that copper stimulates the synthesis of fibronectin in cultures of bovine endothelial cells [27], Suggesting the effects of copper to be more likely to be internal. Fibronectin deposits on the surface of endothelial cells have been an important factor for tracking and forming an adherent endothelium. Later copper has been found to stimulate microvessel formation in the avascular cornea of rabbits, thus dismissing species specificity or uniqueness as a factor in the response. Feeding rabbit diets deficient in copper suppress blood vessels appearance [28]. However, It is been established that copper is one of the causative agents for neovascularization. Copper has been connected with cancer cells in a study that has shown mammary adenocarcinomas having a higher percentage of copper-positive cells; this finding has led Fuchs and de Lustig to postulate a correlation between copper deposits and angiogenic and metastatic ability [29].

Studies also have shown that pro-inflammatory compounds, such as prostaglandin E-1 (PGE-1) and interleukins (IL), stimulate blood vessel formation in animal models. Leukocytes in a stressful or Copper-dependent manner were postulated to release matrix metalloproteinases (MMPs) that result in degradation of extracellular matrix component, collagen, which is believed to assist the movement of pre-existing endothelial cells from a confined connective tissue environment [30]. Copper is an obligatory cofactor of angiogenesis and stimulator of 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 [31]. Copper has been shown to influence the bioactivity or production of a number of angiogenic factors including VEGF-A [32, 14] and shares some of the pathways utilized by hypoxia to regulate VEGF-A expression [15, 32]. Copper containing intra uterine devices (IUD) increases inflammatory action and uterine bleeding [33]. Initial studies have been done on serum, secretory endometrium and menstrual blood copper levels in women using copper-T IUD. There has been no change in the serum copper levels, but the secretory endometrium and menstrual blood copper levels have been significantly increased in the endometrial tissue samples which have been taken after insertion of IUD when compared with samples taken before insertion. With these results it has been concluded that copper is not stored in basal layers of the endometrium but is continually released from the copper IUDs [34]. Thus previously serum copper levels have

been studied only in women using copper-T IUD. The effects of copper and ceruloplasmin on neovascularization in endometrium and metastasis have not been investigated closely, because early research into angiogenesis has focused more on growth factors and cell signaling agents in the response to angiogenic stimulation. Copper has been shown to influence either the bioactivity or the production of a number of growth factors involved in the initiation of angiogenesis, including VEGF [35] basic fibroblast growth factor (β-FGF) and angiogenin. The effect of copper on these factors may arise directly, or it may occur through a more complex, indirect process, but it is not yet clear how the modulation of these factors by copper could fully explain the mechanism involved in differentiation of endothelial cells in comparison to other cell types. Although copper deficiency appears to inhibit an angiogenesis but other cellular processes dependant on copper show no clinical disruption. It is possible that a number of regulatory angiogenic proteins, such as VEGF, FGF and angiogenin are being activated by growth factor-stimulated endothelial-cell-excrete copper [36]. Sen et al. 2002, in their study has shown that copper sulfate induced VEGF expression in primary as well as transformed human keratinocytes at physiologically relevant concentrations. Immunohistochemistry of the wound site has demonstrated increased VEGF in the copper-treated mice compared with saline treated controls [35]. Thus, therapy aimed at depleting copper may be a successful anti-neoplastic strategy which may target multiple angiogenic growth factors. In fact, as outlined below, inhibiting tumor growth via copper depletion has been a successful strategy in animal models [37]. Copper is incorporated in the extra-cellular matrix that forms the very structure of blood vessels. Copper acts as a co-factor to molecules known as β-FGF, VEGF, and angiogenin. Without it, they cannot function, and growth of new blood vessels stops. In other words, copper-reduction blocks angiogenesis by "switching" the endothelial cell into the apoptosis (programmed cell death) pathway, or quiescence, and the cancer remains dormant [38]. Harris et al in 2004 in their study observed that serum levels of proangiogenic factors interleukins (IL)-6,-8, VEGF may correlate with copper depletion but not with disease stability. One must therefore consider that elevating serum copper may be a "cause that favours" rather than "a response against" further tumour development [5].

Role of VEGF on endometrial angiogenesis:

The processes of endometrial differentiation and menstruation involve the remodelling of the endometrial vasculature. The angiogenic factor, vascular endothelial growth factor (VEGF)-A plays an important role in regulation of vascular permeability and the establishment of new blood vessel formation and induces endothelial cell proliferation, migration, differentiation in the endometrium and maintenance of vessel fragility [35]. Although other factors, e.g. epidermal growth factor, transforming growth factor, and platelet-derived growth factor, can also induce neovascular responses these agents are not specific for vascular endothelial cells.

Maturation and remodelling of newly formed micro vessels is accomplished by the coordination of several numerous processes in the

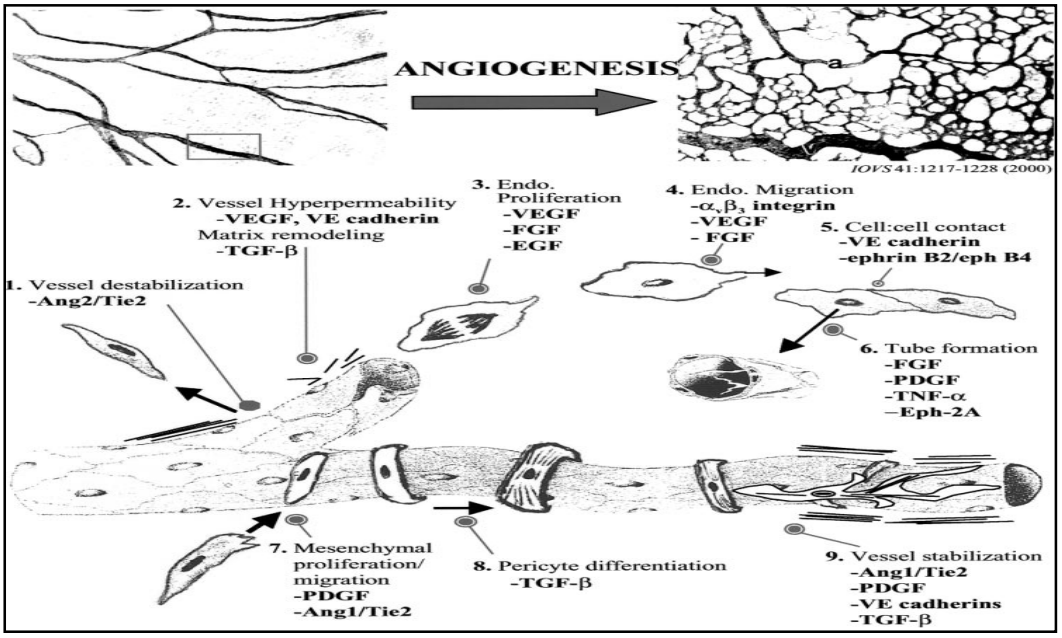

Fig1- Mechanisms of physiological angiogenesis

Normal angiogenesis depends on the coordination of several independent processes. Removal of pericytes from the endothelium and destabilization (1) of the vessel by angiopoietin-2 (Ang2) shift endothelial cells from a stable, growth-arrested state to a plastic, proliferative phenotype. Vascular endothelial growth factor (VEGF)-induced hyperpermeability (2) allows for local extravasation of proteasesand matrix components from the bloodstream. Endothelial cells proliferate (3) and migrate (4) through the remodeled matrix (5), and then they form tubes through which blood can flow (6). Mesenchymal cells proliferate and migrate along the new vessel (7) and differentiate into mature pericytes (8). Establishment of endothelial cell quiescence, strengthening of cell-cell contacts, and elaboration of new matrix stabilize the new vessel (9). TGF-â, transforming growth factor-â; FGF, fibroblast growth factor; EGF, epidermal growth factor; PDGF, plateletderived growth factor; TNF-á, tumor necrosis factor-á; a, arteriole; v, venule

microvasculature that are summarized in Fig.1 [39].

Currently the VEGF family includes VEGF-A, PIGF (placenta growth factor), VEGF-B, VEGF-C, VEGF-D, VEGF-E and svVEGF (snake venom VEGE). Among the VEGF family, VEGF-A has also been localized to individual cells, presumed to be leukocytes, distributed throughout the endometrial stroma. These cells have been identified as neutrophils through dual immunohistochemical staining by Möller et al [40]. Alternative exon splicing of a single VEGF gene results in the synthesis of at least 5 polypeptide isoforms of 121, 145, 165, 189, and 206 amino acids [41]. The active forms of these VEGF glycoproteins are homodimers linked via disulfide bridges [42, 43] and the various isoforms may be further processed by posttranslational mechanisms, e.g. by plasmin. The existence of multiple VEGF species implies that they have different biological properties, distribution, and synthesis. The most widely expressed forms, VEGF 121 and VEGF 165, are freely soluble, while the 189 and 206 species are sequestered and thus remain in the extracellular matrix [44, 45]. An important biological property that distinguishes the different VEGF isoforms, therefore, is their ability to bind to extracellular matrix components, such as heparin and heparan-sulfate. Thus, VEGF 121 lacks the amino acids encoded by exons 6 and 7 of the

gene and binds weakly, if at all, to heparin [46]. The 121 and 165 amino acid isoforms promote permeabilization of blood vessels and proliferation of vascular endothelial cells [47], while VEGF 189 uniquely induces endothelial cell proliferation [45]. Two structurally related vascular endothelial cell specific tyrosine kinase receptors, fms-like tyrosine kinase (flt-1) and kinase domain region (KDR/flk-1), bind VEGF with high affinity [48]. KDR/flk-1 appears essential for endothelial cell differentiation, while flt-1 may be involved in vascular assembly [49, 50]. The physiological importance of the VEGF flt-1/KDR/flk-1 receptor system in blood vessel formation is based on several classical studies showing that: (1) the spatiotemporal expression of VEGF and its receptors correlates closely with angiogenesis in various systems [44]; (2) antibodies to VEGF or flk-1 [51-52] or administration of truncated soluble flt-1 receptor to rats [53] or marmoset monkeys [54] block angiogenesis; (3) targeted inactivation of the VEGF gene in mice resulted in disruption of vasculogenesis and induced embryonic lethality [55, 56]; and (4) mice lacking flt-1 or KDR/flk-1 die in early development because of the absence of vasculogenesis [49, 50]. VEGF-A expression has also been reported in uterine macrophages in the secretory phase of the cycle. Production of VEGF-A and the up-regulation of macrophage numbers premenstrually may implicate these cells in the menstrual process, where hypoxia and VEGF could lead to an induction of MMPs, and also in the revascularization of the endometrium after menstruation [57]. Heavy menstrual blood loss has been associated with aberrations in the synthesis and production of vasodilatory prostanoids from the uterus. In women diagnosed with menorrhagia, PGE_2 synthesis and prostaglandin E binding sites are greater in uterine tissues compared with normal women and correlate directly with menstrual blood loss. Prostaglandin I_2 (prostacyclin) and nitric oxide synthesis are also elevated in menstrual blood collected from women with menorrhagia [57]. This suggests that the degree or duration of menstrual bleeding in women diagnosed with menorrhagia may be augmented after elevation of vasodilatory factors. Elevation of these vasodilatory factors may further enhance menstrual bleeding and vascular dysfunction by up-regulating the COX/prostaglandin biosynthetic pathway via a positive feedback loop and promoting an autocrine / paracrine up-regulation of growth factors specific for vascular function (such as VEGF) [58]. The presumed over expression of COX enzymes in menorrhagia and the established role of COX enzymes in regulation of pro- and antiangiogenic factors would suggest a disturbance in the balance of these factors in menorrhagia compared with normal menstruation [57].

Conclusion:

Angiogenesis is a complex process that relies on the coordination of many different activities in several cell types. Endothelial cells, pericytes, fibroblasts, and immune mediators express many different cytokines and growth factors that react with other cells or extracellular matrix components to effect endothelial cell migration, proliferation, tube formation, and vessel stabilization. Under physiological conditions, angiogenesis is a highly ordered process required for the normal

remodelling of the primary vascular plexus formed during vasculogenesis. Not only are chemical mediators such as cytokines and membrane proteins essential for remodelling the vascular tree, but biomechanical forces such as blood flow and shear stress are also critical angiogenic factors. The reliance of a newly formed vessel on a flow of blood for survival ensures that only those vessels supporting a physiological function become a part of the vascular network. Because any non-functional component is eliminated from the system early in development, such a mechanism is an extremely efficient use of resources, space, and energy. There are many endogenous and exogenous factors involved in mechanism of angiogenesis; among those factors copper is having a significant contribution in etiology of angiogenesis in tumour and dysfunctional uterine bleeding condition.

Copper stimulation of cell growth has been known for a long time and the underlying mechanism involved is to link copper stimulation of VEGF expression [59, 32]. Thus, cellular copper levels may affect the synthesis of proteins by enhancing or inhibiting the transcription of specific genes [60]. Ceruloplasmin may function as an antioxidant in two different ways. Free copper and iron ions are powerful catalysts of free radical damage. By binding copper, ceruloplasmin prevents free copper ions from catalyzing oxidative damage [61]. Serum copper levels and ceruloplasmin levels may fall to 30% of normal in cases of severe copper deficiency. One of the most common clinical signs of copper deficiency is an anemia that is unresponsive to iron therapy but corrected by copper supplementation. The anemia is thought to result from defective iron mobilization due to decreased ceruloplasmin activity [60]. However, It is been demonstrated that copper stimulation of human umbilical vein endothelial cells (HUVEC), cell growth at the physiologically relevant level is not VEGF-dependent, but is eNOS-dependent. This provides a novel insight into the understanding of copper promotion of angiogenesis and copper chelation-induced regression of angiogenesis. It appears that VEGF is required for the growth of endothelial cells, but eNOS is more responsible for the stimulation of cell growth above normal. Copper is required for VEGF expression and is also able to increase VEGF production [4]. In conclusion, copper has been identified as an exogenous factor playing a roles in regulating angiogenesis, copper has been shown to have an effect on production of number of angiogenic factors including VEGF-A [23, 24]. Copper and copper complexes have shown to directly stimulate angiogenesis in several animal model systems while copper chelation has shown to inhibit angiogenesis [36]. Copper containing Intra Uterine Devices (IUD) increases inflammatory action and uterine bleeding [62]. It has been hypothesized that elevated serum copper and VEGF levels could be associated with menorrhagia, which is excessive menstrual bleeding in the absence of a well defined pelvic pathology, often referred to as Dysfunctional Uterine bleeding [31].

Copper at a physiologically relevant level stimulates growth of HUVECs in cultures. This stimulation is not accompanied by increases in VEGF production and anti-VEGF antibody neutralization does not inhibit copper

stimulation of cell growth; thus, the cell growth stimulating effect of copper is not VEGF-dependent. On the other hand, copper at the same level increases eNOS production and gene silencing of eNOS inhibit copper recovery of TEPA inhibition of cell growth, indicating that the copper effect is eNOS-dependent. This observation is in contrast to current belief that cell growth stimulated by copper is VEGF-dependent and provides novel insights into angiogenesis promoted by copper.

References:

1. Folkman J. Tumor angiogenesis: therapeutic implications. *N Engl J Med* 1971; 285 (21):1182-1186.
2. Hiroko Kodama and Chie Fujisawa. Copper metabolism and inherited copper transport disorders: molecular mechanisms, screening, and treatment. *Metallomics* 2009; (1): 42-52.
3. Brem S, Wotoczek-Obadia MC. Regulation of angiogenesis by copper reduction and penicillamine: antagonism of cytokine and growth factor activity. AACR Special Conference, Orlando, Fla. *Angiogenesis and Cancer Research* 1998:24-28.
4. Turnlund JR. Copper. In: Shils ME, Shike M, Ross AC, Caballero B, Cousins RJ, eds. Modern Nutrition in Health and Disease. 10th ed. Philadelphia: Lippincott Williams and Wilkins; 2006:p286-299.
5. Harris ED. Copper. In: O'Dell BL, Sunde RA, eds. Handbook of nutritionally essential minerals. New York: Marcel Dekker, Inc; 1997:p231-273.
6. ACOG Committee on Practice Bulletins-Gynecology, American College of Obstetricians and Gynecologists. ACOG Practice Bulletin: Management of anovulatory bleeding. *Int J Gynaecol Obstet* 2001; 72 (3):263-271.
7. Kenneth M, Wells FM. Recent Advances in Histopathology. *Advances in Endometrial Pathology* 2001; 19:17-35.
8. Hickey M, Pillai G, Higham JM, Sullivan M, Horncastle D, Doherty D, Stamp G. Changes in endometrial blood vessels in the endometrium of women with hormone replacement therapy-related irregular bleeding. *Hum Reprod* 2003; 18 (5):1100–1106.
9. Shizukuda Y, Tang S, Yolota R, Ware J A. Vascular endothelial growth factor-induced endothelial cell migration and proliferation depend on a nitric oxide-mediated decrease in protein kinase C delta activity. *Circ Res* 1999;85(3):247-256.
10. Funovics P, Brostjan C, Niqisch A, Fila A, G rochot A, Mleczko K, Was H, Weiqel G, Dulak J, Jozkowicz A. Effects of 15d-PGJ(2) on VEGF-induced angiogenic activities and expression of VEGF receptors in endothelial cells. *Prostaglandins Other Lipid Mediat* 2006; 79(3-4):230-244.
11. Zheng ZZ, Liu ZX. Activation of the phosphatidylinositol 3-kinase/protein kinase Akt pathway mediates CD151-induced endothelial cell proliferation and cell migration. *Int J Biochem Cell Biol* 2007; 39(2):340-348.
12. Polytarchou C, Papadimitriou E. Antioxidants inhibit human endothelial cell functions through down-regulation of

endothelial nitric oxide synthase activity. *Eur J Pharmacol* 2005; 510(1-2):31-38.

13. Huang LE, Gu J, Schau M, Bunn HF. Regulation of hypoxia-inducible factor-1alpha is mediated by an O_2-dependent degradation domain via the ubiquitin-proteasome pathway. *Proc Natl Acad Sci USA* 1998; 95(14):7987–7992.
14. Tanimoto K, Makino Y, Pereira T, Poellinger L. Mechanism of regulation of the hypoxia-inducible factor-1 alpha by the von Hippel-Lindau tumor suppressor protein. *EMBO J* 2000; 19 (16):4298-4309.
15. Li Q, Chen H, Huang X, Costa M. Effects of 12 metal ions on iron regulatory protein 1 (IRP-1) and hypoxia-inducible factor-1 alpha (HIF-1alpha) and HIF-regulated genes. *Toxicol Appl Pharmacol* 2006; 213(3):245-255.
16. Yuan Y, Hilliard G, Ferguson T, Millhorn DE. Cobalt inhibits the interaction between hypoxia-inducible factor-alpha and von Hippel-Lindau protein by direct binding to hypoxia-inducible factor-alpha. *J Biol Chem* 2003; 278 (18):15911-15916.
17. Salnikow K, Donald SP, Bruick RK, Zhitkovich A, Phang JM, Kasprzak KS. Depletion of intracellular ascorbate by the carcinogenic metals nickel and cobalt results in the induction of hypoxic stress. *J Biol Chem* 2004; 279(39):40337-40344.
18. Martin F, Linden T, Katschinski DM, Oehme F, Flamme I, MukhopadhyayCK, Eckhardt K, Tröger J, Barth S, Camenisch G, Wenger RH. Copper-dependent activation of hypoxia-inducible factor (HIF)-1: implications for ceruloplasmin regulation. *Blood* 2005; 105 (12):4613-4619.
19. Jiang Y, Reynolds C, Xiao C, Feng W, Zh ou Z, Rodriguez W, Tvaqi SC, Eaton JW, Saari JT, Kang YJ. Dietary copper supplementation reverses hypertrophic cardiomyopathy induced by chronic pressure overload in mice. *J Exp Med* 2007; 204(3):657–666.
20. Feng W, Ye F, Xue W, Zhou Z, Kang YJ. Copper regulation of hypoxia-inducible factor-1 activity. *Mol Pharmacol* 2009; 75(1):174-182.
21. Hu GF. Copper stimulates proliferation of human endothelial cells under culture. *J Cell Biochem* 1998; 69(3):326–335.
22. Li S, Xie H, Li S, Kang YJ. Copper stimulates growth of human umbilical vein endothelial cells in a vascular endothelial growth factor-independent pathway. *Exp Biol Med (Maywood)* 2012; 237(1):77-82.
23. Sen CK, Khanna S, Venojarvi M, Trikha P, Ellison EC, Hunt TK, Roy S. Copper-induced vascular endothelial growth factor expression and wound healing. *Am J Physiol Heart Circ Physiol* 2002; 282(5):H1821-1827.
24. Harris ED. A requirement for copper in angiogenesis. *Nutr Rev* 2004; 62(2):60-64.
25. Girling JE, Rogers PA. Recent advances in endometrial angiogenesis research. *Angiogenesis* 2005; 8(2):89-99.
26. McAuslan BR, Reilly W. Endothelial cell phagokinesis in response to specific metal

ions. *Exp Cell Res* 1980; 130(1):147-157.

27. Hannan GN, McAuslan BR. Modulation of synthesis of specific proteins in endothelial cells by copper, cadmium, and disulfiram: an early response to an angiogenic inducer of cell migration. *J Cell Physiol* 1982; 111(2):207-212.
28. Ziche M, Jones J, Gullino PM. Role of prostaglandin E1 and copper in angiogenesis. *J Natl Cancer Inst* 1982; 69 (2):475-482.
29. Fuchs AG, de Lustig ES. Localization of tissue copper in mouse mammary tumors. *Oncology* 1989; 46 (3): 183–187.
30. Lin MT, Chen YL. Effect of copper ion on collagenase release. Its implication in corneal vascularization. *Invest Opthomol Vis Sci* 1992; 33(3): 558–563.
31. Rafi A, Ramkrishna D, Sabitha K, Mohanty S, Pragna Rao. Serum copper and vascular endothelial growth factor (VEGF) in dysfunctional uterine bleeding. *American Journal of Biochemistry and Molecular Biology*; 2011 (3):284-290.
32. Wenger RH. Cellular adaptation to hypoxia: O_2 sensing protein hydroxylases, hypoxia-inducible transcription factors, and O_2 regulated gene expression. *FASEB J* 2002; 16(10): 1151-1162.
33. Burbos N, Musonda P, Giarenis I, Shiner AM, Giamougiannis P, Morris EP, Nieto JJ. Predicting the risk of endometrial cancer in postmenopausal women presenting with vaginal bleeding: the Norwich DEFAB risk assessment tool. *Br J Cancer* 2010; 102(8): 1201-1206.
34. Fahmy K, Ghoneim M, Eisa I, el-Gazar A, Afifi A. Serum and endometrial sodium and potassium levels with inert and copper containing IUCDs and relation to serum steroid levels. *Contraception* 1992; 45(6):573-581.
35. Sen CK, Khanna S, Venojarvi M, Trikha P, Ellison EC, Hunt TK, Roy S. Copper-induced vascular endothelial growth factor expression and wound healing. *Am J Physiol Heart Circ Physiol* 2002; 282(5):H1821-1827.
36. Finney L, Mandava S, Ursos L, Zhang W, Rodi D, Vogt S, Legnini D, Maser J, Ikpatt F, Olopade OI, Glesne D. X-ray fluorescence microscopy reveals large-scale relocalization and extracellular translocation of cellular copper during angiogenesis. *Proc Natl Acad Sci USA* 2007; 104(7):2247-2252.
37. Goodman VL, Brewer GJ, Merajver SD. Copper deficiency as an anti-cancer strategy. *Endocr Relat Cancer* 2004; 11(2):255-263.
38. Duchette R, Gallant S, Wolf C. Tetrathiomolybdate Copper Reduction Therapy as an Antiangiogenic Treatment for Lymphoma and Other Cancers. http://george-eby-research.com/html/anti_ang.html.
39. Papetti M, Herman IM. Mechanisms of normal and tumor-derived angiogenesis *Am J Physiol Cell Physiol* 2002; 282(5):C947-970.
40. Möller B, Rasmussen C, Lindblom B, Olovsson M. Expression of the angiogenic growth factors VEGF, FGF-2, EGF and their receptors in normal human endometrium during the menstrual cycle.

Mol Hum Reprod 2001; 7(1):65-72.

41. Houck KA, Ferrara N, Winer J, Cachianes G, Li B, Leung DW. The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. *Mol Endocrinol* 1991; 5(12):1806-1814.
42. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* 1989; 246(4935):1306-1309.
43. Gospodarowicz D, Abraham JA, Schilling J. Isolation and characterization of a vascular endothelial cell mitogen produced by pituitary-derived folliculo stellate cells. *Proc Natl Acad Sci U S A* 1989; 86(19):7311-7315.
44. Ferrara N, Davis-Smyth T. The biology of vascular endothelial growth factor. *Endocr Rev* 1997; 18(1):4-25.
45. Houck KA, Leung DW, Rowland AM, Winer J, Ferrara N. Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. *J Biol Chem* 1992; 267(36):26031-26037.
46. Cohen T, Gitay-Goren H, Sharon R, Shibuya M, Halaban R, Levi BZ, Neufeld G. VEGF121, a vascular endothelial growth factor (VEGF) isoform lacking heparin binding activity, requires cell surface heparan sulfates for efficient binding to the VEGF receptors of human melanoma cells. *J Biol Chem* 1995; 270(19):11322-11326.
47. Kondo S, Matsumoto T, Yokoyama Y, Ohmori I, Suzuki H. The shortest isoform of human vascular endothelial growth factor permeability factor (VEGF/VPF 121) produced by *Saccharomyces cerevisiae* promotes both angiogenesis and vascular permeability. *Biochim Biophys Acta* 1995; 1243(2):195-202.
48. de Vries C, Escobedo JA, Ueno H, Houck K, Ferrara N, Williams LT. The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science* 1992; 255(5047):989-991.
49. Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* 1995; 376(6535):62-66.
50. Fong GH, Rossant J, Gertsenstein M, Breitman ML. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature* 1995; 376(6535):66-70.
51. Kim KJ, Li B, Winer J, Armanini M, Gillett N, Phillips HS, Ferrara N. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. *Nature* 1993; 362(6423):841-844.
52. Fraser HM, Dickson SE, Lunn SF, Wulff C, Morris KD, Carroll VA, Bicknell R. Suppression of luteal angiogenesis in the primate after neutralization of vascular endothelial growth factor. *Endocrinology* 2000; 141(3):995-1000.
53. Ferrara N, Chen H, Davis-Smyth T, Gerber HP, Nguyen TN, Peers D, Chisholm V, Hillan KJ, Schwall RH. Vascular

endothelial growth factor is essential for corpus luteum angiogenesis. *Nat Med* 1998; 4(3):336-340.

54. Wulff C, Wilson H, Rudge JS, Wiegand SJ, Lunn SF, Fraser HM. Prevention and intervention by treatment with vascular endothelial growth factor trap A40. *J Clin Endocrinol Metab* 2001; 86(7):3377-3386.
55. Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 1996; 380(6573):435-439.
56. Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ, Moore MW. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* 1996; 380(6573):439-442.
57. Jabbour HN, Kelly RW, Fraser HM, Critchley HO. Endocrine regulation of menstruation. *Endocr Rev* 2006; 27(1):17-46.
58. Clancy R, Varenika B, Huang W, Ballou L, Attur M, Amin AR, Abramson SB . Nitric oxide synthase/COX cross-talk: nitric oxide activates COX-1 but inhibits COX-2-derived prostaglandin production. *J Immunol* 2000; 165(3):1582-1587.
59. Lingen MW. Role of leukocytes and endothelial cells in the development of angiogenesis in inflammation and wound healing. *Arch Pathol Lab Med* 2001; 125(1):67-71.
60. Rafi A, Devaki R, Sabitha K, Mohanty S, Rao P. Importance of Serum Copper and Vascular Endothelial Growth Factor (VEGF-A) Levels in Postmenopausal Bleeding. *Ind J Clin Biochem* 2012; 28 (2):147-151.
61. Johnson MA, Fischer JG, Kays SE. Is copper an antioxidant nutrient? *Crit Rev Food Sci Nutr* 1992; 32(1):1-31.
62. Kulier R, O'Brien PA, Helmerhorst FM, Usher-Patel M, D'Arcangues C. Copper containing, framed intra-uterine devices for contraception. *Cochrane Database Syst Rev* 2007; (4):CD005347.

***Author for Correspondence:** Dr. Pragna Rao, Department of Biochemistry, Kasturba Medical College, Manipal University, Manipal, Karnataka, India Cell:09866397508*
Email: pragna.rao@manipal.edu, drpragnarao@gmail.com