Value of Vascular Endothelial Growth Factor, Nitric Oxide and Endostatin Measurement in Follow up of Cancer Breast Patients

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Abstract: Background & aim: Angiogenesis is an essential early requirement for both tumor growth and dissemination. This study was designed to measure plasma levels of angiogenesis promoters; vascular endothelial growth factor (VEGF) & nitric oxide (NO) and the angiogenesis inhibitor; endostatin (ES) in patients with cancer breast; preoperative, four weeks after operation and after the end of chemo-radio therapy to evaluate their role in follow up of breast cancer. Also, we aimed to study the correlation between hormonal receptor states of the tumor and these parameters. **Patients and methods:** This study was performed on twenty two female patients with second stage cancer breast and eighteen apparently healthy females as controls. Both patients and controls were classified according to the menopausal state into pre-menopausal (nine patients and nine controls) and postmenopausal (thirteen patients and nine controls). Patients were sub grouped according to the time of operation and treatment into; preoperative, postoperative and after-treatment groups. All patients and controls were subjected to full clinical examination, chest X-ray, mammography & abdominal ultrasound examination. Bone scan was done for patients only. Histopathological types and assessment of estrogen and progesterone receptors status were done for all sample slides. Routine laboratory investigations and plasma VEGF, NO and endostatin were estimated for all patients and controls. The previous clinical and laboratory investigations were done for all patients during the follow up period. **Results:** Plasma VEGF, NO and ES levels were significantly elevated in both preoperative groups of patients (pre- and post-menopausal) compared to their respective controls. Postoperatively, plasma levels of VEGF and NO were significantly reduced in pre- and post-menopausal patients compared to their preoperative levels respectively. Whereas, compared to control groups, plasma VEGF levels were significantly elevated in both pre- and post-menopausal patients while NO showed significant elevation only in post-menopausal patients. ES levels showed significant elevation in pre- and post-menopausal patients compared to their respective preoperative and control levels. After chemo-radiotherapy, plasma levels of VEGF in pre- and post-menopausal patients were significantly reduced compared to their respective postoperative levels. However, its level in postmenopausal patients was significantly higher when compared to its respective control level. NO levels showed insignificant difference in pre- and post-menopausal patients compared to their respective postoperative levels but, when compared to control groups, it showed significant elevation. Endostatin levels were reduced in pre- and post-menopausal patients compared to postoperative levels but, its reduction was significant only in postmenopausal group. Compared to control groups, ES levels in both pre- and post-menopausal patients still had significantly elevated levels. There was significant positive correlation between preoperative levels of VEGF and both ES & NO. Also, significant positive correlation was found between preoperative NO and ES levels. Moreover, plasma VEGF level showed significant positive correlation with NO level in postoperative group, and significant negative correlations with ES levels in both postoperative and after-treatment groups. Postoperative VEGF level revealed significant elevation in estrogen receptor (ER) & progesterone receptor (PR) positive patients compared to ER & PR negative patients **Conclusion:** Elevated levels of VEGF and NO after the end of chemotherapy necessitate the addition of anti-angiogenic therapy which would also be beneficial for cases positive for ER and PR to prevent development of hormonal resistance. High endostatin level may be considered a good prognostic marker after completion of therapy in breast cancer patients.

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1.Introduction

Angiogenesis is a microvascular process of formation of new blood vessels out of pre-existing capillaries. It is an important physiological process for supply of oxygen, nutrients, growth factors, hormones, and proteolytic enzymes (Hildreth, 2009). Carcinoma of the breast is the most common cause of cancer mortality among women worldwide.

Therefore, early diagnosis and monitoring of breast cancer is an important priority. Breast cancer is angiogenesis dependent for tumor growth, invasion and metastasis. It is potentially treatable by anti-angiogenic therapy (Hyder, 2006). Several pro-angiogenic growth factors and endogenous inhibitors of angiogenesis have been identified in breast cancer which include vascular endothelial growth factor (VEGF), nitric oxide (NO) and endostatin (ES) (Nazan *et al.,* 2003 and Zhao *et al.,* 2004).

VEGF has a pivotal role in tumor angiogenesis. It increases vascular permeability which provides nutrients to tumors, assists the entry of tumor cells into the vascular system and facilitates invasion and metastasis of tumor cells (Ferrara, 2009 and Recchia *et al.,* 2013). It stimulates proliferation of vascular endothelial growth factor receptor-2 (VEGFR-2) positive tumor cells. Furthermore, VEGF induces the expression of the anti-apoptotic protein BCL-2 or up-regulating the expression of BCL-2 in endothelial cells as well as in breast cancer cells, which promotes survival of tumor cells (Liang *et al.,* 2006 and Recchia *et al.,* 2013).

Nitric oxide is a highly reactive gas that is produced by many tissues. NO has both protumor and antitumor activities (Pervin *et al.,* 2007). NO production is a part of the angiogenic switch in tumor development. It controls angiogenesis by modulating the activity of VEGF released by tumor cells (Pervin *et al.,* 2008).

Endostatin, a cleaved fragment of collagen XVIII, which is mainly localized in the basement membrane zones of the vessels, is a specific potent endogenous angiogenesis inhibitor. Elevated serum endostatin levels have been reported in various types of neoplasms and related to the prognosis in some tumors (Brideau *et al.,* 2007). Endostatin blocks VEGF mediated signaling, via a direct interaction with the kinase-insert domain containing VEGFR-2 (Kim *et al.,* 2002). It prevents VEGF- induced phosphorylation, of VEGFR-1 and VEGFR-2 and inhibits VEGF- induced endothelial cell migration (Swidzinska *et al.,* 2005). ES induces apoptosis and affects levels of anti-apoptotic protein such as BCl-2 inside the cells (Yokoyama *et al.,* 2000). The use of angiogenesis inhibitor as ES in combination with radiation therapy should help to overcome the limitations of each leading to enhanced efficacy and diminished toxicity (Senan and Smit, 2007).

Breast cancer cells contain receptors for estrogen or progesterone that is determined by the genetic status of the tumor (Heer *et al.,* 2001). Women have better prognosis if their tumors are receptor-positive because these cells grow more slowly than receptor-negative cells and respond to hormonal therapy to decrease the effect of estrogen on cancer cells (Weaver and Buckner, 2006). Significant correlations were found between VEGF level in breast cancer patients and the status of estrogen receptor (ER) and progesterone receptor (PR) which may reflect an estrogenic regulation of VEGF in some patients (Dabrosin *et al.,* 2005). Those with positive receptors might benefit from a combination of hormonal therapy withanti-angiogenic treatment (Kut *et al.,* 2007). Bentrari *et al.* (2005) demonstrated a correlation between iNOS expression and the presence of progesterone receptors and that expression of iNOS and the associated high production of NO induce cell death which is mainly due to apoptosis. Also, they reported a correlation between endothelial nitric oxide synthase (eNOS) and ER in breast tumors. On the other hand, Glynn *et al.* (2010) found that NO induces IL-8 only in ER-negative breast cancer cells and IL-8 leading to stem cell invasion, angiogenesis and metastasis. Differences in the tumor microenvironment between ER-positive and ER-negative tumors have been observed that may affect NO signaling (Chavey *et al.,* 2007). Moreover, it was reported that estradiol and tamoxifen have the ability to modulate MMP-2/MMP-9 activity which is associated with increased endostatin levels in ER-positive and PR-positive human breast cancer (Nilsson, 2007).

This study aimed to assess circulating plasma levels of angiogenesis promoters (VEGF & NO) and the angiogenesis inhibitor (ES) in patients with cancer breast preoperative, postoperative and after chemo-radiotherapy to evaluate their roles in follow up of breast cancer patients. Also, we aimed to study the correlation between hormonal receptor status of the tumor and these parameters.

2. Patients and methods

This study was performed on twenty two patients with second stage breast cancer (duct carcinoma) (Tl-2 N1-2MO)recruited from General Surgery Department, Assiut University Hospital. Their ages ranged from 30 to 70 years with mean ±SE of 47±2.3. The study also included eighteen apparently healthy females as a control group. Their ages ranged from 32 to 60 years with mean ±SE of 46±1.9 years.

According to menopausal state, patients and controls were divided into pre-menopausal (nine & nine females) and postmenopausal (thirteen & nine respectively) groups. Patients were classified into: pre-operative, post-operative and after treatment groups.

The following cases were excluded from the study: diabetic patients, patients with benign mass or other histopathological types other than duct carcinoma, and patients with abnormal liver or kidney function tests.

Careful history, clinical examination, chest X-ray, abdominal ultrasonography, ultrasonic mammography, and laboratory investigations were done for all patients and controls. Bone scan was done for all patients. All patients underwent modified radical mastectomy in General Surgery Department. Histopathological types were determined by the pathological examination (proved as duct carcinoma) and assessment of estrogen & progesterone receptors status were done for all tissue sample slides. All patients received postoperative chemotherapy 6 cycles of: Cyclophosphamide, Methotrexate and 5-Fluorouracil (CMF) followed by radiotherapy.

Blood samples were taken from all patients preoperative and follow-up was performed by clinical evaluation and laboratory investigations after 4 weeks of the operation and after adjuvant treatment. Peripheral hemogram was performed on Micros 60. Plasma and serum were stored in aliquots at -20°C until analysis. Serum glucose, kidney and liver function tests were done on Hitachi911 autoanalyser.

Measurement of plasma VEGF level was done by using Human VEGF solid phase Enzyme Linked Immuno Sorbent Assay (ELISA) technique which recognizes both natural and recombinant Hu VEGF-165 (Biosource international Inc, USA, cat. No; KHGO111). Measurement of plasma Endostatin level was done by Neogen's Competitive ELISA technique using Neogen Corporation Product (catalog number; 410010). Plasma nitric oxide level was measured by colorimetric detection of nitrite as azo dye product of the Griess reaction using Assay Designs NO assay kit (catalog number; 917-010).

Statistical analysis

Data entry and analysis were done using SPSS software v.17 (Chicago, USA). Continuous values were described by mean and standard error. Univariate analysis for determining the difference of laboratory variables between the studied groups was performed using *Student's t*-test for continuous variables. Correlations among the studied variables were tested by Spearman's correlation coefficient. Differences were considered statistically significant if *P* value was less than 0.05.

3.Results

Table (1) shows the plasma levels of VEGF, NO & ES in all patient groups compared to control group and to each other. Plasma VEGF, NO and ES levels were significantly elevated in both preoperative groups of patients (pre- and post-menopausal) compared to their respective control levels. Plasma VEGF showed significant reduction in both postoperative groups compared to their respective preoperative groups, whereas compared to control group; it showed significant elevation. In after-treatment group, both pre- and postmenopausal patients showed significant reduction of VEGF level compared to their respective postoperative groups. Whereas, compared to control groups, it showed significant elevation only in postmenopausal group.

There was significant reduction of NO level in both postoperative groups (pre- and postmenopausal) compared to their respective preoperative groups. But, compared to respective control group, only postmenopausal females showed significant elevation of NO. In after-treatment group, no significant difference were found in NO levels in both pre- and postmenopausal patients compared to their respective postoperative levels. In contrast, when compared to control group, both pre and postmenopausal patients showed significant elevation in NO levels.

Endostatin level showed highly significant elevation in both postoperative groups (pre- and postmenopausal) compared to their respective preoperative levels. In addition, ES levels were reduced in both after-treatment groups (pre- and postmenopausal) compared to their respective postoperative groups. But, this reduction was significant only in postmenopausal group of patients and their levels were still significantly higher in both groups (pre- and postmenopausal) when compared to their respective control groups.

There was significant positive correlation between preoperative levels of VEGF and both ES ($r = 0.471$, $P<0.05$) & NO ($r = 0.271$, $P<0.05$), (Figure 1). Also, significant positive correlation was found between preoperative NO and ES levels ($r = 0.382$, $P<0.05$). Moreover, in postoperative group, plasma VEGF level showed positive correlation with NO levels ($r = 0.415$, $P<0.05$) and significant negative correlation with ES levels ($r = -0.357$, $P<0.05$), (figure 2).also, in after-treatment samples, significant negative correlation was found between VEGF and ES levels ($r =- 0.281$, $P<0.05$, respectively),

Regarding tissue hormonal receptors, all the studied pre-menopausal patients were ER negative, two were PR positive, and seven were PR negative. In postmenopausal patients, seven were ER positive, six were ER negative, six were PR positive and seven were PR negative. When classified according to estrogen and progesterone receptors, pre-menopausal groups were not sufficient for statistical evaluation. However, postmenopausal patients showed elevation of VEGF levels in ER & PR positive patients compared to ER & PR negative patients and this elevation was statistically significant in postoperative group only (254±20.3 vs 156±16.6 pg/ml, $P<0.01$ & 256±23 vs 168±19.2 pg/ml, $P<0.01$, respectively). But, no significant difference was found when comparing the levels of NO & ES between ER & PR positive and negative patients in all groups of the studied patients.

Table (1): plasma VEGF, nitric oxide and endostatin levels in preoperative, postoperative and after-treatment groups (pre- and post-menopausal patients) compared to control groups and to each other

	Preoperative group		Postoperative group		After-treatment group		Control group	
	Pre-menopausal (No=9)	Post-menopausal (No=13)	Pre-menopausal (No=9)	Post-menopausal (No=13)	Pre-menopausal (No=9)	Post menopausal (No=13)	Pre Menopausal (No=9)	Post Menopausal (No=9)
VEGF (pg/ml)	409.5±98**	499.2±59**	155.5±26**	209.2±19**	52.2±18	62.3±12.5*	22.2±8.7	24.4±8
Nitric oxide (µmol/L)	144.4±7.2**	137.3±4**	64.1±6	80±4.5*	78.8±4*	77.4±6.4*	59.1±3.1	56.2±4.7
Endostatin (ng/ml)	34.8±7.5*	53.3±10**	69.3±12**	78.5±14**	50.4±12**	40.7±8*	16.4±4.1	15.2±5

P value of comparison between the studied patient groups:
- Post operative versus preoperative (pre- and postmenopausal) VEGF, NO, ES; $P<0.001$.
- Pre-menopausal after treatment versus postoperative VEGF; $P<0.001$ & NO, ES; $P>0.05$.
- Postmenopausal after treatment versus post operative VEGF, ES; $P<0.001$ & NO; $P>0.05$

Data were presented as mean±SE* $P<0.05$, ** $P<0.01$ (each group versus the respective control group).

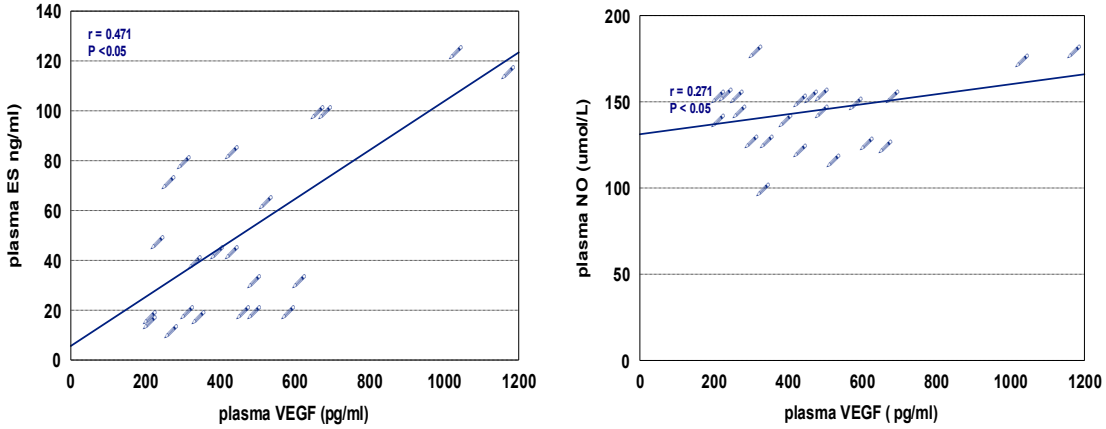

Figure (1): Correlation between VEGF and both endostatin (ES) and nitric oxide (NO) in preoperative group.

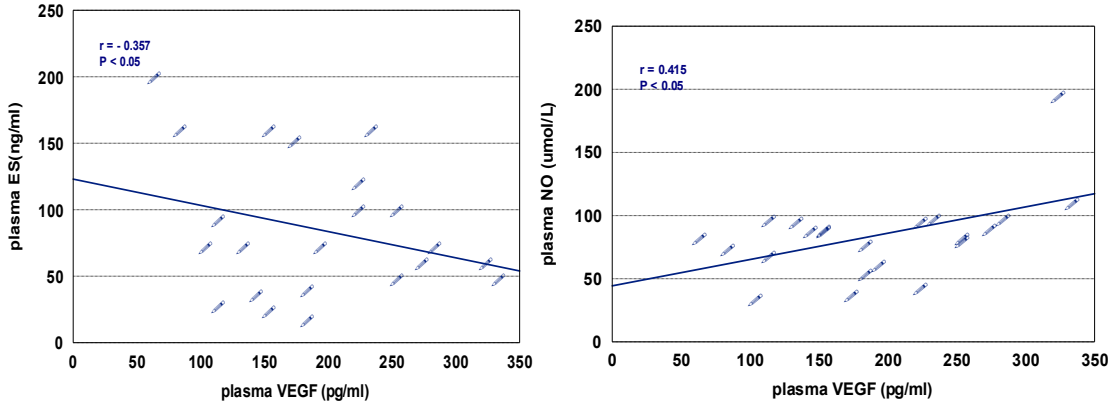

Figure (2): Correlation between VEGF and both endostatin (ES) and nitric oxide (NO) in postoperative group.

4.Discussion

Angiogenesis plays an essential role in breast cancer development, invasion, and metastasis (Hyder, 2006). It is tightly controlled by a balance between pro-angiogenic, and anti-angiogenic growth factors (angiogenic switch) (Gupta and Zhang, 2005). In this study, we measured the circulating levels of two pro-angiogenic factors: Vascular Endothelial Growth Factor (VEGF) & Nitric Oxide (NO) and one anti-angiogenic factor: Endostatin (ES) in patients with second stage breast cancer pre-operative, post-operative (after about four weeks) and at the end of treatment (chemotherapy and radiotherapy).

All the studied patients had elevated preoperative plasma VEGF levels and this elevationwas statistically significant compared with control group. Previous studies by Zhao *et al.*, (2004), Konukoglu *et al.* (2007), Lowery *et al.* (2008) and Recchia *et al.* (2013) also reported this elevation. It was presumed that VEGF has been secreted by cancer cells, tumor infiltrating inflammatory cells and tumor-associated stroma into circulation and act on endothelial cells to promote angiogenesis (Clarke and Sharma, 2006). Also, hypoxia and hypoxia-inducible factor associated with breast cancer play a prominent role in increased VEGF level (Granato *et al.,* 2006). Moreover, VEGF may be responsible for the decreased immune competence observed in advanced cancer, through the reduced maturation of dendritic cells that are important antigen-presenting cells. For this reason, prolonged exposure of the immune system to high levels of VEGF may decrease immune response and thus, facilitate tumor growth (Recchia *et al.*, 2013).

During follow up of the studied patients, a significant reduction of plasma VEGF level was observed in postoperative groupcompared to pre-operative group. These results supported further the hypothesis that the high level of VEGF in the circulation was attributed to the secretion of VEGF from the tumor and show dramatic decline in VEGF after tumor resection (Lowery *et al.*, 2008).

Although the plasma level of VEGF decreased dramatically in the studied patients post operatively, its level is still significantly higher than control level which could be explained by the role of angiogenesis in normal wound repair. Mediators of wound angiogenesis including VEGF, produced by macrophages and platelets, are involved in this angiogenic response to generate nutrients to injured tissue and are central to the development of granulation tissue framework for wound closure (Hormbery *et al.*, 2003).

After the end of adjuvant treatment (chemotherapy and radiotherapy), plasma VEGF level significantly decreased in both pre- and post-menopausal patients compared to their respective post-operative levels, which may be an indicator of the controlled disease status and the treatment-induced response or stabilization, the tumor angiogenesis seems to change into an anti-angiogenic direction (Tang *et al.*, 2007). Also, Ria *et al.* (2004) reported that chemotherapy decreases circulating VEGF level by attenuation of surgery stimulated tumor cell proliferation and blocks endothelial cell growth factor induced neovascularization. So, plasma VEGF level could therefore be a promising candidate for monitoring response to chemotherapy in advanced breast cancer (Granato *et al.*, 2006). Radiotherapy decline VEGF levels through affecting endothelial cell survival & proliferation, cell surface molecule expression and apoptosis (Caine *et al.*, 2007).

However, after radio and chemotherapy, plasma VEGF level was significantly higher in postmenopausal patients compared to control level which may imply new transformations and resistance to radiotherapy (Wu *et al.*, 2002). Another hypothesis is that VEGF may be induced by stress chemotherapy which is genotoxic stress could induce expression of VEGF and VEGF receptors in human cancer cells. Postmenopausal patients may be responsive to angiogenesis inhibitors or anti-VEGF when added to chemotherapy (Fan *et al.*, 2008).

Plasma nitric oxide (NO) level was found to be elevated in all pre-operative samples of the studied patients and its level was significantly elevated compared to control level. This elevation could result from a generalized increase in NO synthesis in both stromal and tumor cells of the breast, inflammatory cells and non immune cell types or reflect increased NO degradation promoted by oxidative stress (Konukoglu *et al.*, 2007). Moreover, upregulation of inducible NO synthase (iNOS) can lead to increase NO production to initiate NO-dependent signal transduction and induce modifications of proteins, lipids and DNA. Aberrant expression of iNOS has been observed in many types of tumors including breast cancer which is associated with markers of poor outcome (Ambs and Glynn, 2011). Elevation can also be explained by upstream signals elicited by VEGF and other proangiogenic factors binding to their receptors which stimulate eNOS activity by regulating its phosphorylation (Roberts *et al.*, 2007). In the present study, significant positive correlations have been found between NO and VEGF level in pre and post-operative groups. This is in agreement with Konukoglu *et al.* (2007). NO production is a part of the angiogenic switch in tumor development. It controls angiogenesis by modulating the activity of VEGF released by tumor cells. Breast cancer cells that over express VEGF require the NO pathway to promote angiogenesis, increase vascular permeability and induce endothelial cell proliferation (Previn *et al.*, 2008).

In this study, plasma nitric oxide decreased significantly in postoperative samplescompared to pre-operative levels. NO could be secreted by cancer cells of the breast. So, by removal of the mass, circulating NO will decrease significantly (Kilic *et al.*, 2006). Beevi *et al.* (2004) reported that; biopsy samples from human breast cancer showed the presence of greater expression of inducible nitric oxide synthase (iNOS) and increased NO degradation promoted by oxidative stress, and removal of the mass associated with decrease in the circulating

NO.After theend of adjuvant treatment (Chemo-radiotherapy), both pre and post menopausal groups showed insignificant difference in NO level compared to post operative level. However, this level was significantly increased compared to control level. Irradiation dose-dependently induced the activation of the pro-angiogenic NO pathway in endothelial cells through increase in endothelial nitric oxide synthase abundance and phosphorylation (Sonveaux *et al.*, 2003). Bhownick and Girotti (2011) reported that following irradiation, there is substantial upregulation of both the cytoprotective iNOS mRNA & NO in breast cancer cells and this cytoprotective effects could reduce treatment efficacy and point to pharmacologic intervention with iNOS inhibitors. Ozyurt *et al.* (2008) reported that patients whose serum NO levels at the end of chemo-radiotherapy were lower than post-operative levels had longer survival than patients with increased NO levels.

Regarding plasma endostatin, the present study revealed significantly elevated levels in pre-operative samplescompared to controls. Cancer cells and host tumor stroma microenvironment which are known to produce a variety of angiogenic stimulators may also be capable of producing a spectrum of angiogenesis inhibitors as endostatin as an attempt to make an angiogenic balance. There is a balance between plasma endostatin and plasma VEGF which determine the rate of tumor growth through the regulation of endothelial cell apoptosis (Hong *et al.*, 2006). High levels of endostatin may inhibit the growth of distant micrometastasis by directly affecting the tumor cells and not just acting viaendothelial cells and blockage of angiogenesis (Yoon *et al.*, 2004).

Endostatin can also display pro-angiogenic activity by elevating cytosolic NO production in endothelial cells and evoking vascular relaxation. In this study, positive correlation was found between ES and NO levels in pre-operative patients. This finding would be in line with Wenzel *et al.* (2006). Post-operativeplasma endostatin levels were significantly elevated in both pre- and postmenopausal patients compared to their respective pre-operative levels. The source of post-operative endostatin is related to the process of wound healing. At four weeks after surgery, the active wound healing process has subsided but collagen turnover and remodeling continues. Endostatin generation is at least partly mediated through protease enzymatic activities, such as elastase and cathepsin L (Teh *et al.*, 2004). In the studied patients, plasma levels of ES&VEGF showed significant positive correlation in pre-operative group which is in agreement with Zhao *et al. 2004*...while significant negative correlation between the two parameters was observed post operatively. After the tumor was removed and serum VEGF decreased significantly, the circulating endostatin was still high implying a new angiogenesis balance was formed. The resultant anti-angiogenesis balance should be beneficial for prognosis of breast cancer. Breast cancer patients with elevated endostatin levels following surgery showed a lower risk of relapse (Zhao *et al.*, 2004).

In this study, plasma endostatin decreased significantly in postmenopausal group of patients after the end of adjuvant treatment (Chemo-radiotherapy), when compared to the post-operative levels, but its level was still significantly higher in pre- and postmenopausal women compared to their respective controls. The reduction of ES level after treatment may be related to the blocking effect of radiation on local angiogenesis. Also, the adjuvant chemotherapy which is traditionally used induce a high level of cytotoxic activity on the rapidly proliferating cells, so lowered level will be expected (Retsky *et al.*, 2004). Radiotherapy decreases the production of this angiogenesis inhibitor from primary tumors (Chung *et al.*, 2006).

According to tissue ER & PR, plasma level of VEGF was higher in ER & PR positive patients compared to those negative for the receptors but this elevation was statistically significant only in postoperative samples of postmenopausal patients. But, no statistically significant difference was found when comparing the levels of NO and ES between ER & PR positive and negative patients in all groups of the studied patients.

It was demonstrated that angiogenic turnover is regulated by estrogen and progesterone, which modulate the expression of VEGF in breast cancer cells (Coradini *et al.*, 2003). It was reported that total VEGF expression is related to poor prognosis in ER-positive patients rather than ER- negative patients (Bando *et al.*, 2005).VEGF has been implicated in the development of hormone- resistance in ER - positive breast cancer. Exogenous VEGF blocked the anti proliferative effects of the anti-estrogen agents or treatment and promote estrogen- independent growth (Stein *et al.*, 2009).

In lights of these results, we conclude that pharmacological intervention with VEGF and iNOS inhibitors would be beneficial for patients who still having high levels of VEGF and NO after completion of adjuvant therapy. Higher levels of VEGF in ER &PR positive postmenopausal patients is implicated in development of hormonal resistance in those patients and necessitate the addition of anti-angiogenic therapy for proper treatment. Sustained high level of ES after completion of therapy makes it useful as a good prognostic marker**.**

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