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EVALUATION OF THE OXIDATIVE STRESS, ANTIOXIDANTS AND TRACE METALS IN EGYPTIAN WOMEN WITH BREAST TUMOR

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ABSTRACT

The increasing global incidence of breast cancer emphasizes the need to understand the various mechanisms involved in breast tumorigenesis. The present study was carried out to determine the variation in level of antioxidants, lipid peroxidation and in blood of untreated patients with malignant and benign breast tumor and compare these levels according to the presence and absence of metastasis. Also, evaluate the prognostic significance of tissue trace elements correlation with the progression of the tumor. A highly significant increase in the level of malondialdehyde, catalase, nitrite (as an index of nitric oxide production) and trace elements were observed while there are significant decreases in the level of superoxide dismutase, glutathione-S transferase and reduced glutathione.

Key words: *oxidative stress, Breast cancer, Trace elements, Antioxidants.*

INTRODUCTION

Cancer has recently become one of the most obsessing issues in the world, since both its incidence and impact on world economy has become enormously huge. Breast cancer is a potentially life-threatening malignancy that develops in one or both breasts (Ghafoor *et al.*, 2003). Most types of tumors that

form in the breast are benign. Although benign breast tumors are abnormal growths, they do not grow uncontrollably or spread, and are not life threatening.

Reactive oxygen species (ROS) universally appear in human environment and are generated in tissues during metabolic and inflammatory processes. They can act at several stages in malignant transformation inducing permanent DNA sequence changes (Zowczak *et al.*, 2001). ROS has been implicated in the pathogenesis of certain diseases, including cancers (Yeha *et al.*, 2005).

Certain antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) can protect against cellular and molecular damage (Singh *et al.*, 2003). Disruption of this delicate between the free radicals and the antioxidants may cause cellular damage and trigger carcinogenesis (Sinah *et al.*, 2009).

A number of important metabolic processes involve enzymes and vitamins containing metal atoms like Mn, Fe, Cu, Zn etc., for example, element Cobalt in vitamin B12, iron in hemoglobin, zinc in carboxypeptidase A, calcium in thermolysin, etc. (Reddy *et al.*, 2003).Many different evidences indicate that various metals act as catalysts in the oxidative damage of biological macromolecules, and therefore, the toxicity associated with these metals may be due, at least in part, to their ability to generate free radicals. It may be assumed that trace metals influence the mechanisms that are responsible for the development of cancer because of their fundamental importance in a number of biochemical and physiological processes in humans (Bower *et al.*, 2005).

The current study was designed to investigate the oxidative status in Egyptian women with cancerous and non-cancerous breast tumor. Also, the level of some trace metals was detected.

SUBJECTS AND METHODS

Subjects and sampling: Seventy-seven Egyptian women with adenocarcinoma breast tumor and 50 subjects with benign breast tumor, aged from 38 to 55 year-old, were selected from the Oncology Center, Mansoura University. Twenty healthy subjects, with no prior history of breast disease and have the same age range, were selected from the same area and served as control group. Patients who had not undergone any previous treatment for their tumors were chosen. The patients were not using hormones, oral contraceptives, and were all nonsmokers. None of them had concomitant diseases such as diabetes mellitus, liver disease or rheumatoid arthritis.

The breast cancer patients were classified into: patients without metastasis (36 patients) and patients with metastasis in their lymph nodes (41 patients). An approval for the study was obtained from the ethical committee of Mansoura University, and written informed consents were obtained from all participants of the study.

Fresh tumor and adjacent normal tissues, 5 cm away from the malignant tumors, were obtained from patients immediately after surgery. The tissues were washed with ice-cold normal saline three times, surrounded fats were trimmed carefully. For trace metals analysis, portions of the tissues were fixed in 10% formalin, and kept in refrigerator until use.

Fasting peripheral blood samples were collected from the patients just before the surgery and from the healthy controls into EDTA tubes. The GSH level was

measured in whole blood. The plasma were separated by centrifugation at 3000 rpm for 10 min., aliquoted and stored at – 20°C for further biochemical assays as lipid peroxidation and nitric oxide determination as well as antioxidant enzymes activities.

Determination of lipid peroxidation: Malodialdehyde (MDA), as the end product of lipid peroxidation, was determined in plasma according to the method of Ohkawa *et al.* (1982). Briefly, 0.2 ml of the sample was mixed with 1.5 ml of 0.05 mol/l HCl and 0.5 ml of 0.67% TBA, and then boiled in water bath at 95°C for 30 min. After cooling, the products were extracted in 2 ml of 15% butanol and centrifuged at 2500 rpm for 30 min at 4°C. The pink-colored chromogen formed was read at 535 nm.

Determination of Superoxide dismutase activity: SOD activity was assayed by the method of Dechatelet *et al.* (1974). The assay mixture in a total volume of 3.0 ml contained 1.8 ml of sodium pyrophosphate buffer, 0.1 ml of plasma, 0.5 ml of nitroblue tetrazolium and 0.5 ml of 0.47 mM NADH, and 0.1 ml of 0.093 mM phenazine methosulfate. The reaction was initiated by the fast addition of phenazine methosulphate and the increase in absorbance at 560 nm due to the formation of reduced nitroblue tetrazolium was followed.

Determination of catalase activity: Catalase activity was assayed by the method of Aebi (1984). The reaction was initiated by the addition of 0.5 ml of 30 mM hydrogen peroxide to the reaction mixture containing 0.5 ml of 50 mM phosphate buffer (pH 7.0) and 0.1 ml of plasma sample. The decrease in absorbance (A) was followed at 240 nm for 30 seconds.

Determination of GSH level: GSH level was determined by mixing 0.5 ml of whole blood with 4 ml of 0.08 N H_2SO_4 .After 10 minutes of standing at room temperature, 0.5 ml of tungstate solution was added to the clear brown

hemolysate. Then the solution was centrifuged at 2000 rpm for 10 minutes and then 2 ml of the filtrate were mixed with 2.5 ml of the tris buffer and 0.2 ml of the DTNB reagent (0.04 g%). After that the absorbance was read at 412 nm (Beutler *et al.,* 1963).

Determination of GST activity: GST activity was measured according to the method of Habig *et al.* (1974) using 1-chloro-2, 4-dinitrobenzene as substrate. The formation of GSH-CDNB conjugate was monitored by the change in absorbance at 340 nm for each sample against its blank.

Determination of nitric oxide: Plasma nitrite concentration was measured using the Griess reagent according to the method of Green *et al.,* (1932). Griess reagent, the mixture (1:1) of 0.1% N-(1-napthyl) ethylenediamine and 1% sulphanilamide in 5% phosphoric acid, gives a red-violet diazo dye with nitrite, and the resultant color was measured at 540 nm.

Determination the concentration of some trace metals: Trace metal analyses have performed in the frozen breast tissues. The concentrations of copper, zinc, iron and manganese in the final solution have been measured at wavelengths of 324.8, 213.4, 248.3 and 283.3 A, respectively, by using single-beam Perkin-Elmer atomic absorption spectrophotometer with a single slit burner and an air acetylene flame. The intensity of absorption can be directly correlated with the amount of the element present in the original sample according to Rosner and Gorfien (1968).

Statistical analysis: Data were expressed as mean value ± standard deviation (SD). Differences between groups were assessed by *t*-test analysis of variance using the Excel software (Microsoft). Statistical significance at $P<0.05$ was considered significant. Correlation coefficient (r) was done by using GraphPad InStat program version 5.02 produced by

GraphPad Software Inc. The Correlation is considered to be significant at $P < 0.05$.

RESULTS

The levels of oxidative status in patients with breast tumor and control subjects are shown in table (1). The level of MDA was measured in the plasma as an indicator for lipid peroxidation. As shown in table (1), MDA was significantly increased in patients with either cancerous or non-cancerous tissues when compared to that of controls ($P<0.05$). The activities of antioxidant enzymes, SOD, and GST, in plasma of breast tumor patients were significantly lower ($P<0.05$) than those of the control group. Also, the level of NO and CAT were found to be significantly increased in breast tumor patients ($P<0.05$) as compared to that of controls. Results also showed that GSH level is significantly reduced ($P<0.05$) in breast tumor patients compared to that of the control subjects.

Table (2) shows the oxidative potential capacity in breast cancer patients with or without metastasis. The level of plasma MDA was significantly increased in metastatic breast cancer patients ($P<0.05$) as compared to non-metastatic breast cancer patients. The activities of SOD, and GST were found to be significantly reduced in metastatic breast cancer patients ($P<0.05$) as compared to those without metastasis. There were significant increase ($p<0.05$) in plasma NO and CAT levels in breast cancer patients with metastasis compared to those without metastasis. There was a significant decrease ($p<0.05$) in blood GSH in breast cancer patients with metastasis compared to breast cancer patients without metastasis.

Results illustrated that the concentrations of tissue trace elements Cu, Zn and Fe in breast cancer patients were significantly higher than that in the adjacent normal and benign breast tumor patients, ($P < 0.05$ and $P < 0.05$), while there

was no significant increase or decrease in Mn level between the study groups ($p>0.05$) (Table 3).

Results illustrated that there was a significant increase in Cu, Zn and Fe concentrations in tissue of metastatic breast cancer patients as compared with non-metastatic breast cancer patients as shown in table 4.

As shown in table 5 and 6, there were a positive correlation ($p<0.05$) between Fe and Cu, Fe and Zn, Cu and Zn, MDA and Cu, MDA and Zn and MDA and Fe in breast cancer patients, also there is a positive correlation ($p<0.05$) between Fe and Zn in normal adjacent tissue. There were a negative correlation ($p<0.05$) between SOD and Cu, SOD and Zn, and SOD and Fe, MDA and SOD.

Table 1: Comparison of free radical and antioxidant levels in study and control groups

Parameter	Control group	Benign group	Malignant group
MDA(nmol MDA/ml)	0.59 ± 0.11	0.88 ± 0.22 [a]	1.4 ± 0.2 [a,c]
GSH(mmoles/L cell)	1667.8 ± 176.3	1526.8 ± 141.1 [a]	1053.6 ± 240.6 [a,c]
SOD (%)	58.86 ± 3.44	49.8 ± 4.7 [a]	21.4 ± 4.5 [a,c]
CAT (µmol H_2O_2/Sec/ml)	0.27 ± 0.06	0.34 ± 0.08 [a]	0.42 ± 0.06 [a,c]
GST(µmol/min/ml)	3.59 ± 0.22	2.94 ± 0.81 [a]	2.53 ± 0.38 [a,c]
NO (µmol/L)	11.85 ± 2.20	15.5 ± 1.85 [a]	19.9 ± 2.4 [a,c]

([a]) Significant against control group ($P<0.05$).
([c]) Significance against benign group ($P<0.05$).

Table 2: Comparison of free radical and antioxidant levels in breast cancer sub-groups

Parameter	Breast cancer without metastasis	Breast cancer with metastasis
MDA(nmol/ml)	1.26 ± 0.08	1.59 ± 0.19 [b]
GSH (mmoles/L cells)	1150.6 ± 242.38	956.4 ± 206.3 [b]
SOD (%)	24.36 ± 1.80	19.61 ± 4.68 [b]
CAT (µmol H_2O_2/Sec/ml)	0.37 ± 0.02	0.46 ± 0.06 [b]
GST(µmol/min/ml)	2.71 ± 0.42	2.39 ± 0.27 [b]
NO(µmol/L)	18.78 ± 1.31	20.59 ± 2.38 [b]

(b) Significant against breast cancer sub-group without metastasis (P <0.05).

Table 3: Tissue trace elements concentration (µg/g tissue) in study groups

Parameter	Benign group	Malignant group	
		Adjacent normal tissue	Breast cancer
Cu	0.84 ± 0.28	0.65 ± 0.20	3.07 ± 0.99 [a,c]
Zn	14.86 ± 2.36	13.85 ± 4.54	20.71 ± 3.71 [a,c]
Mn	0.25 ± 0.100	0.25 ± 0.09	0.30 ± 0.11
Fe	6.56 ± 1.16	3.67 ± 0.95	10.32 ± 2.05 [a,c]

([a]) Significant against adjacent normal tissue (P <0.05).
([c]) Significance against benign group (P <0.05).

Table 4: Tissue trace elements concentration (µg/g tissue) in breast cancer sub-groups

Parameter	Breast cancer without metastasis	Breast cancer with metastasis
Cu	2.35 ± 0.62	3.79 ± 0.67 [b]
Zn	18.00 ± 0.67	23.22 ± 3.60 [b]
Mn	0.26 ± 0.096	0.31 ± 0.122
Fe	8.89 ± 1.03	11.65 ± 1.83 [b]

(b) Significant against breast cancer sub-group without metastasis (P <0.05).

Table 5: Pearson correlation coefficients between elemental concentrations and DNA contents in breast cancer patients and their normal adjacent tissue.

	Fe/Cu	Fe/Zn	Cu/Zn
Normal adjacent	---	0.53*	---
Breast cancer	0.52*	0.42*	0.47*

*** Correlation is significant for P <0.05.**

Table 6: Pearson correlation coefficients between elemental concentrations, oxidants and antioxidant in breast cancer patients.

	SOD/Cu	SOD/Zn	SOD/Fe	MDA/Cu	MDA/Zn	MDA/Fe	MDA/SOD
Breast cancer	-0.53*	-0.38*	-0.42*	0.57*	0.54*	0.60*	-0.32*

* Correlation is significant for P <0.05.

DISCUSSION:

The incidence of breast cancer appears to be increasing world-wide (Althius et al., 2005). In Egypt, breast cancer is the most common cancer among women, representing 18.9% of total cancer cases (35.1% in women and 2.2% in men) according to the National Cancer Institute (NCI) data (Omar et al., 2003).

Lipid peroxidation was one of the most important expressions of oxidative stress (Katalinic et al., 2005) because it amplified the free radical production process. Higher plasma MDA levels were found in patients with both benign and malignant breast tumor compared to healthy control subjects in this study. MDA levels was also increased with progression of breast cancer.

In agreement with increased lipid peroxidation found in blood of patients with breast tumor, Subramaniam et al. (1994) have reported an increased serum and erythrocyte MDA levels in breast cancer patients. Wang et al. (1996) demonstrated that MDA can accumulate in human breast tissues and reach relatively high levels in the breast tissues of women with breast cancer.

Elevation of lipid peroxidation in cancer may be due to defective antioxidant system which leads to the accumulation of lipid peroxides in cancer tissue which are released into the blood stream (kumaraguruparan et al., 2002a). It has been claimed that MDA acts as a tumor promoter and co-carcinogenic agent because of its high cytotoxicity and inhibitory action on protective enzymes.

SOD and CAT are considered primary antioxidant enzymes, since they are involved in direct elimination of reactive oxygen metabolites (Halliwell, 2006). In our study SOD level was found to be low in all tumor patients as compared with control. The comparison between subgroups in breast cancer

patients showed a decreased in the level of SOD with the progression of malignancies. Sinha et al. (2009) has demonstrated in their respective studies that the reduction in SOD activity increases the toxic effect of $O_2^{\cdot}$ and this might lead to severe cellular damage. The SOD level might have risen initially to meet the challenge of carcinogenesis before final consumption which might occur in the late stages of this disease. Kasapovic´ et al., (2008) found that the suppression of blood cell Cu/ZnSOD activity related to breast cancer is due to post-translational chemical modification of this enzyme.

Glutathione, as a reductant, is very important in maintaining the stability of erythrocyte membranes (Pastore et al., 2003). The lower GSH levels seen in the present study support the hypothesis that the glutathione status is inversely related to malignant transformation. These findings are in agreement with the results of Kasapovic´ et al. (2008) and Kumaraguruparan et al. (2005) who reported that a positive correlation has been observed between enhanced synthesis of GSH and high rates of cell proliferation in tumors .

Glutathione-S-transferase (GST) is an antioxidant enzyme which has a protective role against oxidative stress (Fiander and Schneider, 1999). In the current study, the activity of GST was decreased significantly in the plasma of benign and malignant breast tumor patients when compared with that of control group. This observation is in agreement with the result of Kumaraguruparan et al. (2002b) who found a tendency for blood GST to decrease with cancer progression. The depletion of GSH may be responsible for the decreased activity of GPx and GST in benign and malignant breast tumor patients.

$NO^{\cdot}$ is a multifunctional molecule that is implicated in a wide variety of physiological and pathological processes. $NO^{\cdot}$ is readily oxidized to nitrite and nitrate in biological systems. It exhibits a dual role in tumor progression, as it can act as a promoter or antitumor promoter, depending on its concentration (Eijan et

al., 2002). In this study, we demonstrated that in breast cancer, plasma level of nitric oxide is significantly increased compared to benign breast tumors and control subjects and its level in plasma was increased with the progression of malignancies.

NO˙ reacts rapidly with superoxide anion to form peroxynitrite anion, which may be cytotoxic by itself or easily decompose to the highly reactive and toxic hydroxyl radical and nitrogen dioxide (carr et al., 2000).

Go¨nenc et al. (2006) found that the increased nitrate and nitrite levels in serum and tissue of breast carcinoma compared with those of benign breast disease are shown by the increased expression of NO synthase activity. Enhanced production of nitric oxide in phagocytic cells may occur to protect them from oxidative stress.

In the present study, higher levels of iron in the malignant breast tissues relative to the normal and benign tissues are observed and this level increase with the progression of tumor. This observation is well supported by most of the earlier works (Kuo et al. 2002 and Pasha et al., 2008). Elevated concentrations of this metal would be associated with vascularity and increased blood supply to a growing tumor, acting as a regulatory factor for angiogenesis (Le and Richardson, 2002). This elevation can also be associated with presence of iron in oxidative processes which take place in neoplastic tissues (acting as a catalyst in the conversion of hydrogen peroxide to free-radical ions) (Majewska et al., 2007).

The concentration of copper in the carcinoma tissues of breast is observed to be higher than that in the normal and benign tissues and this level increase with the presence of metastasis. This observation being well supported by Ebrahim et al. (2007), Geraki et al. (2004) and Siddiqui et al. (2006).

However, this metal level elevation can be justified once copper is involved in oxidative enzymatic reactions, which produce hydroxyl radicals that adversely modify proteins and other important organic molecules causing breast cancer (Tapia et al., 2003) and it is also an important factor for angiogenesis (Brem et al., 1998).

According to the findings of the current study, a significant high level of zinc was found in cancerous tissue as compared to that of either normal or benign breast tissues. Furthermore, zinc level was found to be highly significant in metastatic cancer when compared to that of non-metastatic cancer. Zinc is an essential element for the immune system (Rink et al., 2001).

Elevated concentrations of this metal possibly contribute to the development of breast cancer since zinc is necessary for cell proliferation and tumor growth (Majewska et al., 2007), also zinc can play an important role as activator of matrix metalloproteinases (MMPs), a family of structurally and functionally related endoproteinases that are involved in the degradation of the extracellular matrix (ECM), contributing to tumor cell invasion and metastasis (Duffy et al., 2000).

The observed correlation between Fe and Cu and between Cu and Zn in breast cancer tissues is consistent with the fact that these elements contribute to angiogenesis process promoting new blood vassels formation and contributing to tumor growth and dissemination (Konemann et al., 2005). Moreover, this result emphasizes the function of copper as an important cofactor for the metabolism of the iron (Nasulewiez et al., 2004).

Correlations in normal adjacent and breast cancer tissue were found for Fe and Zn that can be related with increase of metabolic activity and angiogenic starting process in adjacent tumor tissues, beyond a regulated transport of Fe and

Zn through the mammary gland epithelium, as suggested by Domellof et al. (2004).

Our findings showed that a positive correlation was observed between tissue Cu, Zn, Fe and serum MDA levels in breast cancer patient but not in benign group. We suggest that increased oxidative stress present in breast cancer may result from changes in the levels of certain trace elements.

CONCLUSION

Increased nitrite levels in plasma of breast carcinoma compared with those of benign breast tumor are shown by the increased expression of NO synthase activity. Enhanced production of nitric oxide in phagocytic cells may occur to protect them from oxidative stress.

The deficiency of SOD, GSH and GSH-dependent enzymes in the circulation of breast cancer patients may be due to increased utilization to scavenge lipid peroxides as well as sequestration by tumor cells. An interesting observation in the present study is that the changes in circulatory lipid peroxides and antioxidants seen in breast cancer patients were also evident in benign breast tumor patients placing them in a "high-risk" category. Cancer disturbs the physiological functions of biological cells which are manifested in the variation of trace elements concentrations in benign, malignant and normal tissue obtained from the same subject and this indicates the possibility of using these elements as discriminant factor. Moreover, for prognostic purposes, the high levels of these elements may serve as early indicator of cancer disease.

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تقييم جهد الأكسدة ومضادات الأكسدة والعناصر النادرة لدى النساء المصريات المصابات بأورام في الثدي

تهدف هذه الدراسة الى بحث إجهاد الاكسده عن طريق قياس معدل الاكسده الفوقية للدهون(المتمثل في ثنائي الديهيد المالونات) ومضادات الاكسده (المتمثلة في قياس إنزيم السوبر اوكسيد ديسميوتيز والكتلاز والجلوتاثيون ترانسفيراز و ماده الجلوتاثيون) وأكسيد النيتريت في المرضى المصابين بأورام حميدة و أورام خبيثة في الثدي. تهدف هذه الدراسة أيضا إلى قياس العناصر النادرة في عينات النسيج السرطاني والنسيج المصاب بأورام حميدة في الثدي.ولتحقيق هذا الهدف فقد أجريت الدراسة على ١٢٧ مريضه مصابين بأورام حميدة وخبيثة في الثدي وتم تقسيمهم إلى مجموعتين:

١ـ مجموعة المريضات المصابات بأورام حميدة وتشمل هذه المجموعة ٥٠ مريضة.

٢ـ مجموعة المريضات المصابة بأورام خبيثة وعددهم ٧٧ وتم تقسيمهم على حسب انتشار المرض في الغدد الليمفاوية أو عدم انتشاره إلى:

١ـ٣٦ مريضه فى حاله سلبيه من الانتشار.

٢ـ ٤١ مريضه فى حاله موجبه للانتشار.

كما تم اخذ عينات من مجموعه تضم ٢٠ من الأصحاء كمجموعه ضابطه للمقارنة.

تم أجراء التحاليل التالية في مصل الدم:

قياس معدل الاكسده الفوقية واكسيد السوبر اوكسيد ديسميوتيز والكتلاز والجلوتاثيون ترانسفيراز وماده الجلوتاثيون.تم إجراء التحليل التالي على عينات النسيج:

قياس تركيز العناصر النادرة .

ولقد توصلت الدراسة للنتائج التالية:

-ارتفاع في مستوى ثنائى الادهيد المالونات واكسيد النيتريت فى الدم وكذلك ارتفاع تركيزات العناصر النادرة في النسيج السرطانى.

-انخفاض فى مستوى إنزيم السوبر اوكسيد ديسميوتيز والكتلاز وانزيم الجلوتاثيون ترانسفيراز وماده الجلوتاثيون.ونستخلص من نتائج هذه الدراسه:

- القياس المبكر لمعدل الاكسده الفوقيه للدهون ومحاوله تفاديها باستخدام مضادات الاكسده تعتبر من أهم الاحتياطات لمنع تدهور هذا المرض.
- زيادة تركيزات العناصر النادرة يمكن استخدامه في تشخيص مدى تقدم المرض وفى التفرقه بين النسيج الحميد والخبيث .