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ORIGINAL ARTICLE

Oxidative Stress and Anti-oxidants in Pre and Post-operative Cases of Breast Carcinoma

Operasyon Öncesi ve Sonrası Meme Kanseri Olgularında Oksidatif Stres ve Antioksidanlar

Sohail HUSSAIN[1,2]*, Mohammad ASHAFAQ[2]

[1]Aligarh Muslim University, J.N. Medical College, Department of Biochemistry, Uttar Pradesh, India
[2]Jazan University, Faculty of Pharmacy, Department of Pharmacology and Toxicology, Jazan, Kingdom of Saudi Arabia

ABSTRACT

Objectives: To investigate the existence of oxidative stress in the sera of patients with breast cancer and its effects on the consequent breast cancer.

Materials and Methods: This study included 50 control volunteers, 50 patients with breast cancer, and 50 patients with post-operative breast cancer. Patients with pre-operative cancer were clinically and histopathologically diagnosed for breast carcinoma with stage 0, not having therapeutic history. The control 50 healthy female volunteers had the same socio-economic status, and no history of any cancer. After obtaining consent, venous blood was collected from the volunteers by vein puncture using a 10 mL sterile disposable syringe and needle. About 8 mL of blood was collected, 4 mL of which was poured into a heparinized bulb and 4 mL was allowed to clot. The levels of MDA, NO, GSH, and activities of RBC-SOD (in RBC lysate), NOS, copper and zinc GPx, and CAT, and vitamins A, C, and E metabolites were measured in the sera of each group.

Results: The activities of RBC-SOD and the levels of MDA, NO, as well as the NOS were significantly higher in the sera of all patients with breast cancer as compared with the controls. However, the levels of GSH and vitamins A, C, and E, as well as the activities of copper and zinc GPx and CAT were decreased in patients with breast cancer when compared with the controls.

Conclusion: The study provides further evidence for the presence of oxidative stress in the serum of patients with breast carcinoma. Patients with higher levels of MDA showed deficiencies of antioxidants and trace elements in the serum. A poor dietary antioxidant status and high oxidant levels are associated with the risk of breast cancer, thus suggesting that patients with breast cancer should take nutritive supplements to balance the antioxidant and oxidant levels for better outcomes.

Key words: Breast cancers, oxidants, antioxidants

ÖZ

Amaç: Meme kanserli hastaların serumlarında oksidatif stresin varlığını ve meme kanserine etkilerini araştırmaktır.

Gereç ve Yöntemler: Bu çalışmaya 50 gönüllü kontrol, 50 meme kanseri hastası ve 50 ameliyat sonrası meme kanseri hastası dahil edildi. Bu hastalar, klinik ve histopatolojik olarak evre 0 meme kanseri teşhisi olan ve tedavi almamış hastalardır. Kontrol grubundaki 50 sağlıklı kadın gönüllü aynı sosyo-ekonomik statüye sahipti ve hiçbir kanser öyküsü yoktu. Onam alındıktan sonra, 10 mL'lik steril tek kullanımlık bir şırınga ve iğne kullanılarak venöz ponksiyon ile gönüllülerden venöz kan alındı. Yaklaşık 8 mL kan toplandı, 4 mL'si heparinize bir ampul içerisine alındı ve 4 mL'si pıhtılaşmaya bırakıldı. Her grubun serumunda MDA, NO, GSH ve RBC-SOD (RBC lizat), NOS, bakır ve çinko GPx ve CAT aktiviteleri, A, C ve E vitaminleri ölçüldü.

Bulgular: Meme kanseri olan tüm hastaların serumlarında RBC-SOD aktiviteleri, MDA, NO ve NOS düzeyleri, kontrollere göre anlamlı olarak daha yüksektir. Bununla birlikte, kontrol grubu ile karşılaştırıldığında meme kanseri olan hastalarda GSH, A, C ve E vitaminleri ile bakır ve çinko GPx ve CAT aktiviteleri azalmıştır.

Sonuç: Bu çalışma, meme kanseri hastalarının serumunda oksidatif stresin varlığına yönelik ileri kanıt sunmaktadır. Serumunda daha yüksek seviyelerde MDA bulunan hastaların antioksidan ve eser elementlerinin yetersiz olduğunu göstermiştir. Zayıf antioksidan diyet ve yüksek oksidan düzeyleri meme kanseri riski ile ilişkilidir, bu nedenle meme kanseri olan hastaların daha iyi sonuçlar için antioksidan ve oksidan seviyelerini dengeleyecek besleyici takviyeler almaları gerektiğini düşündürmektedir.

Anahtar kelimeler: Meme kanseri, oksidanlar, antioksidanlar

*Correspondence: **E-mail:** shussainamu@gmail.com, **Phone:** +90 557632511 **ORCID-ID:** orcid.org/0000-0001-6958-6191
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INTRODUCTION

Breast cancer is one of the most common malignant tumors in women with unknown etiology.[1] Reactive oxygen species (ROS) such as superoxide anion radical ($\downarrow O_2^-$), hydroxyl radicals ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2) are produced during aerobic metabolism.[2] Levels of free radicals are controlled by anti-oxidant enzymes [catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD)] and anti-oxidants (vitamins E, C, glutathione, carotenoids and flavonoids).[3] Under normal conditions, there is a balance between the activities of anti-oxidant enzymes and intracellular levels of these anti-oxidants. This balance is essential for the survival of organisms and their health. An imbalance between the production and detoxification of ROS results in oxidative stress. ROS has been implicated in the pathogenesis of certain diseases, including cancer.[4,5] It reacts with polyunsaturated fatty acids to induce the release of toxic and reactive aldehyde metabolites such as malondialdehyde (MDA), one of the end products of lipid peroxidation (LPO). MDA may be involved in tumor promotion because it can interact with the functional groups of a variety of cellular compounds.[6] To control the over production of ROS, cells protect themselves against oxidative damage by antioxidant detoxifying mechanisms, which helps to lower ROS concentrations in the body. SOD catalyzes the dismutation of $O_2\downarrow$ into H_2O_2, and CAT is responsible for the detoxification of H_2O_2 to oxygen and water.[7] Glutathione acts as a reducing agent that maintains enzymes in an active state as an antioxidant.[8] The main protective roles of glutathione against oxidative stress are: (i) to act as a cofactor for several detoxifying enzymes such as glutathione reductase and GPx against oxidative stress; (ii) to participate in amino acid transport through the plasma membrane; (iii) to scavenge the ($^{\bullet}OH$) and singlet oxygen, detoxifying the H_2O_2 and lipid peroxides by the catalytic action of GPx, and (iv) to regenerate the most important antioxidants back to their active forms.[8,9] Nitric oxide (NO) acts as an intracellular second messenger and provides an efficient system for cellular regulation, interaction, and defense. Its role strictly depends on the chemical reactivity with oxygen and metals. Recent studies revealed that the involvement of altered NO levels was associated in the pathogenesis of cervical cancer (CaCx).[10] Some findings have shown that concentration of NO higher or lower than the basal level caused a tumorigenic effect in CaCx.[11] In addition to the body defense mechanism, there are vitamins that provide the body with much needed immunity and a mechanism of self-defense to fight against various pathogens. Studies indicate that the level of these antioxidants in the body decrease in carcinogenesis. The level of vitamin E was found to vary in cervical carcinogenesis.[12] Vitamin C has free radical scavenging property, it directly reacts with hydroperoxides and plays an important role in sparing vitamin E. Thus, the role of vitamin C is very important in the treatment of cancer.[12,13] Strong oxidizing agent such as NO, interacts with organic substances and with the support of transition metal like copper which creates more reactive species such as ($^{\bullet}OH$).[14] Zinc is an integral part of biomembranes, it may be involved in the control of membrane integrity, stability, and LPO -related injuries. Zinc plays an inhibitory role in RNA and DNA polymerase, phosphodiesterase, and an activating effect on the membrane-bound enzyme, adenylcyclase, and there is suggested role of zinc in carcinogenesis.[15] The levels of LPO and antioxidant status in patients with breast cancer after surgery remain unknown. To address these issues, the levels of oxidants and antioxidants in the patients with breast cancer were examined during and after tumor removal.

MATERIALS AND METHODS

The present study was conducted in the Department of Biochemistry, Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh, Uttar Pradesh, India. This study included 50 control volunteers, 50 patients with breast cancer, and 50 patients with post-operative breast cancer. Further, the women were within the age group of 35-65 years and were from the same demographic area. Patients with pre-operative cancer were clinically and histopathologically diagnosed for breast carcinoma with stage 0, not having therapeutic history. The 50 healthy female control volunteers were from the same socio-economic status, had no history of smoking, alcoholism, and any cancer. Volunteers/patients with a history of smoking, alcoholism, and other diseases that induce oxidative stress such as diabetes mellitus, pulmonary diseases, and respiratory diseases were excluded from the study. The study was approved by the institutional ethics committee and written informed consent was received from the patients. After obtaining consent, venous blood was collected from the volunteers/patients under aseptic conditions by veinpuncture usinga 10 mL sterile disposable syringe and needle. About 8 mL of blood were collected, 4 mL of which was poured into a heparinized bulb and 4 mL was allowed to clot. Serum and plasma were separated by centrifugation at 3000 rpm for 10 min at room temperature. The plasma pellets were taken as a source of red blood cells (RBCs). The samples were stored at 4°C before analysis and all the samples were analyzed on the day of collection.

Assay of LPO

Measurement of MDA in serum was estimated using the thiobarbituric acid (TBA) method.[16,17] MDA, which is a stable end product of fatty acid peroxidation, reacts with TBA at acidic conditions to form a complex that has a maximum absorbance at 535 nm. A 300 µL sample was mixed with 1.5 mL of 0.05 mol/L HCl and 0.5 mL of 0.67% TBA and then mixed and boiled well in water at (95°C) for 30 min. After cooling, the products were extracted with 2 mL of 15% butanol and centrifuged at 2500 rpm at (4°C) for 30 min. The rate of LPO was expressed as MDA formed per hour per milligram of protein using the molar extinction coefficient of 1.56×10^5 mol/L^{-1} cm^{-1}.

Assay of SOD

SOD activity was estimated using a commercial Ransod kit (Randox Laboratories, UK). This method is based on the generation of $^{\cdot}O_2$ produced by xanthine and xanthine oxidase,

which react with phenyl tetrazolium chloride to form a red formazan dye. RBC-SOD activity was measured in RBC hemolysate through the degree of reaction inhibition. The results are expressed as U/mL. RBC-SOD was assessed using Winterbourn's method, which is based on the ability of SOD to inhibit the reduction of nitroblue tetrazolium by superoxide, which is generated by the reaction of photo-reduced riboflavin and oxygen.[18]

Assay of CAT

CAT activity was measured by monitoring the decrease in absorption of H_2O_2 at 240 nm.[19] One hundred microliters of serum was added to a 0.5 mL quartz cuvette containing 400 µL of 20 mM H_2O_2 in phosphate-buffered saline (25°C) and mixed thoroughly by pipetting. The absorbance was monitored immediately at 240 nm for three minutes at one-minute intervals. CAT activity was measured for each sample and the rate in mAU/min/mg protein was averaged.

Assay of GSH

GSH status analyses were assayed from blood samples obtained through a venous arm puncture and the serum was separated by centrifugation.[20] After the separation, the buffy coat was removed and the packed cells were washed 3 times with physiologic saline. One hundred-microliter aliquots of washed RBCs were added to 300 mL ice-cold 5% metaphosphoric acid. To completely precipitate proteins, the samples were vortexed and incubated on ice for 10 min. After centrifugation at 4°C at 12000 rpm for 10 min, the supernatants were filtered through a 0.2 mm filter and diluted 5 times before being injected into the capillary electrophoresis system.

GPx assay

GPx activity was assayed according to the method of Haque et al.[21] The assay mixture consisted of 0.1 M phosphate buffer (pH 7.4), 1 mM EDTA, 1 mM sodium azide, 1 mM GSH, 0.2 mMNADPH, 0.25 mM H_2O_2, and 0.1 mL sera. Oxidation of NADPH was recorded spectrophotometrically at 340 nm. The enzyme activity was calculated as nanomoles of NADPH oxidized per minute per milligram of protein using a molar extinction coefficient of 6.22×10^3 M^{-1} cm^{-1}.

NO assay

Serum was deproteinized first to convert NO to nitrate, the stable product of NO. The nitrate present in the filtrate was then reduced to nitrite, which was measured by diazotization of sulphanilamide and coupled with naphthylethylene diamine, as in Najawa and Cortas's method.[22]

Inducible NO (iNOS) assay

iNOS synthase activity was measured *in vitro* in blood lymphocytes (suspended in MEM @ 1×10^6 viable cells/mL) using arginine and Greiss reagent with the method of Stuehr and Marletta.[23] The optical density of the citrulline formed was determined spectrophotometerically with a ultraviolet-visible spectrophotometer (Shimazu) at 540 nm against a control.

Assay of vitamins

Serum vitamin C was estimated by the method of Kyaw[24], where phosphotungstic acid was first deproteinized and then reacted with ascorbic acid to produce a blue color. Vitamins A and E were measured using high-performance liquid chromatography (HPLC) as per the modified method of Omu et al.[25]. In brief, α-tocopherol acetate and retinol acetate were pipetted into an Eppendorf tube. Blood serum was added and vortexed; the hexane extract of vitamin A and E was taken out in a glass tube, dried under nitrogen stream, and dissolved into methanol. Finally, this preparation was injected into an HPLC fitted with reverse phase of column C18. The vitamins were eluted with methanol at a flow rate of 1.5 mL/min for 15 minutes. The peak heights and curve areas of vitamin A, E, and acetates were measured to calculate the amount of these vitamins in the serum with an ultraviolet detector at 292 nm.

Assay of trace elements

Copper and zinc in serum were estimated by using an atomic absorption spectrophotometer.

Statistical analysis

The experimental data are expressed as mean±standard deviation. In this study, p values of $p<0.05$ were considered significant. Statistical analysis was performed using the STATGRAPHICS plus statistical package.

RESULTS

The results showed that the level of MDA was increased significantly in all groups of patients with breast cancer as compared with the controls ($p<0.05$). The MDA level was decreased 13.81% in post-operative patients as compared with pre-operative patients. On the other hand, the activity of RBC-SOD was significantly increased in pre-operative and post-operative patients ($p<0.05$) as compared with controls, but its activity in post-operative patients was decreased 34.69% compared with pre-operative patients (Table 1). However, the activity of CAT was decreased significantly in all groups of patients with breast cancer compared with the controls ($p<0.05$), but this activity in post-operative patients was increased 23.13% compared with pre-operative patients (Table 1).

The contents of GSH was increased 23.52% in the post-operative group compared with the preoperative group. The level of NO was decreased 24.32% in the post-operative group when compared with the pre-operative group, and iNOS activity was also decreased 38.01% in the post-operative group as compared with the pre-operative group (Table 1). Significantly decreased RBC-SOD activity ($p<0.05$) and plasma levels of vitamins C, A, and E ($p<0.05$) were observed in all patients with cancer when compared with the healthy controls. It was observed that the concomitant decline in the activity of RBC-SOD and levels of vitamins were associated with the progression of cancer, but the levels of all vitamins were not significant in post-operative patients (Table 2). The Cu/Zn ratio was also found to be significantly ($p<0.05$) lower in post-operative patients when compared with the pre-operative.

Table 1. Serum levels of oxidants and antioxidative enzymes in control and in patients with breast cancer before and after surgery

Parameters	Control	Pre-operative	Post-operative	Percentage change in post-operative group
NO (μM/L)	36.56±6.13	78.34±12.79* (114.44%)	59.28±11.61# (-24.33 %)	↓24.33*
iNOS (nmoles/mL/min)	1.19±0.53	3.92±0.77* (229.41%)	2.43±0.51# (-38.01%)	↓38.01*
MDA (μM/L)	2.13±0.69	3.098±1.02* (45.07)	2.67±0.94# (13.81)	↓13.81
SOD (U/mL)	390.99±58.76	712.43±154.87* (82.21)	465.88±113.57# (34.69)	↓34.69*
RBC-SOD (units/mg)	362513±217.9	2387.34±398.97* (99.24)	2669.48±276.9# (10.56)	↑10.56
GSH (mM/L)	0.64±0.1017	0.39±0.1943* (-39.06%)	0.51±0.3109# (30.77)	↑30.77%
GPx (U/L)	26673.37±3994.56	8304.40±1856* (-68.86)	10234.43±2743# (18.85)	↑18.85
CAT (U/mL)	78.68±8.51	44.98±16.78* (-42.83)	58.52±21.79# (23.13)	↑23.13*

*: Significant changes, pre-oprative vs control, #: Significant changes post-operative vs pre-oprative, ↑: Increase, ↓: Decrease, GPx: Glutathione peroxidase, CAT: Catalase, GSH: Glutathione, RBC-SOD: Red blood cell-superoxide dismutase, MDA: Malondialdehyde, NO: Nitric oxide, iNOS: Inducible nitric oxide

Table 2. Serum levels of vitamins and trace elements incontrols and in patients with breast cancer before and after surgery

	Control	Pre-operative	Post-operative	Percentage change in post-operative group
Vitamin A (mg/dL)	38.76±4.61	32.99±6.33	34.81±4.19	↑5.22**
Vitamin C (mg/dL)	1.84±0.09	0.98±0.08	1.02±0.14	↑39.2**
Vitamin E (mg/dL)	81.2±0.89	6.32±0.91	7.14±0.72	↑11.4**
Serum copper (μg%)	114.04±12.79	168.6±9.89	142.5±8.16	↓15.48**
Serum zinc (μg%)	106.7±9.74	70.92±11.83	81.91±19.11	↑13.41**

**: Significant changes, ↑: Increase, ↓: Decrease

DISCUSSION

Increased oxidative stress and LPO are implicated in carcinogenic processes. The magnitude of this damage depends on ROS levels and on the body defense mechanisms against them, which are mediated by various cellular antioxidants.[8,26] MDA is produced by the oxidation of polyunsaturated fatty acids in membranes induced by free radicals, and is an indicator of oxidative damage. Many studies have examined the possibility of a connection between LPO and cancer.[6,27] Higher plasma MDA levels have been reported in patients with cancer than in controls.[27] However, lower LPO measured in plasma using TBA-reactive substances has also been reported in breast cancer groups compared with controls.[28] Our findings are in agreement with most earlier studies that suggested that there might be some accumulation of ROS, which causes significantly higher LPO at cellular and molecular levels. ROS derived from $NO^{\bullet}$ and released from inflammatory cells. Radical can act on neighboring dividing epithelial cells, leading to somatic mutations in crucial cancer-causing genes.[29] $NO^{\bullet}$ produced in solid tumors has been implicated in enhanced vascular permeability, and increased tumor blood flow, and hence sustained tumor growth.[30] GSH, as a reductant, is very important in maintaining the stability of erythrocyte membranes. It is implicated in the cellular defense against xenobiotics and deleterious compounds such as free radicals and hydroperoxides.[31] GSH in the nucleus also maintains the redox state of critical protein sulphydryls that are necessary for DNA repair and expression.[8] A decrease in blood GSH in circulation has been reported in several diseases including malignancies.[32] The lower GSH levels in patients with breast cancer supports the hypothesis that glutathione status is inversely related in malignant transformation.[33] Several studies have reported decreased levels of GSH in the blood of patients with breast cancer compared with control subjects.[34,35] Our results showed that there were significant decreases in blood GSH levels in patients with breast cancer compared with the control subjects. The decrease in GSH concentration can be explained by decreased GSH synthesis and/or increased GSH consumption in the removal of peroxides and xenobiotics. Cells have strong endogenous antioxidant defenses against increased LPO, ROS, and NO. SOD and CAT are the first line of defense against superoxide and H_2O_2.[36,37] The significant increase in SOD activities indicates the formation of more superoxide radicals and their removal because SOD metabolizes superoxide radicals.[38] Furthermore, the decrease in activity of SOD might be due to an association with free radical generation, which causes damage to enzymes by cross linking or damaging the nuclear DNA, leading to mutations. It may also be due to a scarcity of trace elements such as zinc and manganese, which act as cofactors for this enzyme.[39] However, the significant decrease in CAT activity indicates the toxicity produced by H_2O_2.[27] Studies have shown that oxidants may activate gene expression through antioxidant responsive elements,[40] which explains the enhanced enzyme activities. Our data showed a significant increase in SOD and a decrease in

CAT activities in patients with breast cancer compared with the controls. A substantial increase in GSH level and increase CAT activity were found in the postoperative patients, which might be due to the free radical scavenging property. The decreased levels of vitamin C may be associated with its action as an antioxidant where it gets used. Its synergism with vitamin E and A helps in sparing vitamin E, and during this process, vitamin C gets used,[12,13] which is seen through the significant decline in plasma ascorbic acid. A negative correlation between vitamin C and MDA was noted, thereby leading to the conclusion that free radicals are scavenged by ascorbic acid and thus get utilized. Copper can interact directly with the bases of DNA at G-C sites.[41] The addition of copper to DNA *in vitro* mediates more extensive DNA base damage, inducing more mutations.[42] Copper may also elaborate other free radical species such as •OH; therefore, the inactivation/loss of certain tumor suppressor genes can lead to the initiation and/or progression of carcinogenesis. The elevation in copper levels may be due to mobilization of copper from tissue to serum.[42]

Zinc is used for cell growth and maintains the integrity of the membrane. However, cancerous cells may consume the zinc present in the circulation for tumor growth and maintain membrane integrity.[43] This might be a possible reason for the depletion of zinc in breast cancer. The increased ratio of Cu/Zn is due to the significant decrease in Zn and concomitant increase in copper. Therefore, in the pre-operative group, the ratio of Cu/Zn was increased as compared with the controls. As this ratio is altered, this could be considered as a risk factor for tumor growth or carcinogenesis.

CONCLUSIONS

In conclusion, breast cancer is related to an increase of oxidants in serum with concomitant decrease of antioxidant defense capacity. Overall, our data support the importance of endogenous antioxidant in the etiology of breast cancer across all levels of predicted risk. There are some significant differences in the oxidant and antioxidant status in the blood of patients with breast cancer before and after surgery. Prospective studies in a larger population should be conducted to confirm our present findings.

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