

REVISED MANUSCRIPT

**OXIDATIVE STRESS AND ANTI-OXIDANTS IN PRE AND POST-OPERATIVE
CASES OF BREAST CARCINOMA**

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1. ABSTRACT

Breast cancer is one of the most common types of cancer, which accounts for highest rate of morbidity and mortality in women. One of the mechanisms of breast cancer is the oxidative stress, which plays a crucial role in its pathogenesis. The increased production and ineffective scavenging of oxidants may play a crucial role in tissue damage leading to cancer. In this study, activities of red blood cells-superoxide dismutase (RBC-SOD)(in RBC lysate) and the levels of malondialdehyde (MDA), nitric oxide (NO) as well as the nitric oxide synthase (NOS) were significantly higher in the sera of all breast cancer patients as compared to the control. However, the levels of glutathione (GSH) and vitamins A, C, E as well as the activities of copper and zinc glutathione peroxidase (GPx) and catalase (CAT) were decreased in patients of breast cancer when compared with control. The patients who have shown higher level of malondialdehyde in the serum have shown 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 the breast cancer patients should take nutritive supplements to balance the antioxidant and oxidant levels for a the better outcome.

2. INTRODUCTION

Breast cancer is one of the most common malignant tumors in women with unknown etiology (1). The reactive oxygen species (ROS) such as superoxide anion radical ($\text{O}_2^{\cdot-}$), hydroxyl radicals ($\cdot\text{OH}$) and hydrogen peroxide (H_2O_2) are produced during aerobic metabolism (2). The levels of free radicals are controlled by anti-oxidant enzymes (catalase, glutathione peroxidase, superoxide dismutase) 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, the cells protect themselves against oxidative damage by antioxidant detoxifying mechanisms that help to lower ROS concentrations in the body.

Superoxide dismutase (SOD) catalyses the dismutation of O_2^- into H_2O_2 , while catalase (CAT) is responsible for the detoxification of hydrogen peroxide to oxygen and water (7). Glutathione (GSH) 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 glutathione peroxidase against oxidative stress; (ii) to participate in amino acid transport through the plasma membrane; (iii) to scavenge the hydroxyl radical and singlet oxygen, detoxifying the hydrogen peroxide and lipid peroxides by the catalytic action of glutathione peroxidase (iv) to regenerate the most important antioxidants back to their active forms (8-9). $NO\cdot$ acts as an intracellular second messenger and provide 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 level is associated in the pathogenesis of cervical cancer (CaCx) (10). Some findings have shown that the concentration of NO high or low than the basal level caused tumorigenic effect in CaCx (11). In addition to the body defense mechanism, there are vitamins that provide the body with the 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 important role in sparing vitamin E. So, the role of vitamin-C is very beneficial in the treatment of cancer (12-13). Strong oxidizing agent, interacts with organic substances and with the support of transition metal like copper which creates more reactive species such as $\cdot OH$ (14). Zinc is an integral part of biomembrane, it may be involved in the control of membrane integrity, stability and lipid peroxidation related injuries. Zinc plays an inhibitory role in RNA and DNA polymerase, phosphodiesterase and activating effect on membrane bound enzyme, adenylyl cyclase and suggests the role of zinc in

carcinogenesis (15). The levels of lipid peroxidation and antioxidant status in breast cancer patients after surgery remain unknown. To address these issues, the levels of oxidants and antioxidants in the breast cancer patients were examined during and after tumor removal.

3. MATERIALS AND METHODS

The present study was carried out in Department of Biochemistry, Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh, Uttar Pradesh, India. This study includes 50 control volunteers, 50 patients with breast cancer and 50 patients with post operative breast cancer. Further, the female were within the age group of 35-65 years from same demographic area. They were clinically and histopathologically diagnosed for breast carcinoma with stage 0, not having therapeutic history. The control 50 healthy female volunteers were from the same socio-economic status, having no history of smoking, alcoholism and any cancer. The volunteers/patients having history of smoking, alcoholism and other diseases which induce oxidative stress such as diabetes mellitus, pulmonary diseases, respiratory diseases etc. were excluded from the study. Our study was ethically approved by the institutional committee.

After obtaining prior consent, venous blood was collected from the volunteers/patients under aseptic condition by vein puncture using 10 ml sterile disposable syringe and needle. About 8 ml of blood were collected, out of which 4 ml were poured in 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 pellets of plasma were taken as a source of RBCs. The samples were stored at 4°C before analysis and all the samples were analyzed on the same day of collection.

3.1 ASSAY OF LPO

Measurement of MDA in serum was estimated by 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 maximum absorbance at 535 nm. Sample 300 µl was mixed with 1.5ml of 0.05mol/l HCl and 0.5ml of 0.67% TBA and then mixed and boiled well in heated 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 molar extinction coefficient of $1.56 \times 10^5 \text{ mol/L}^{-1} \text{ cm}^{-1}$.

3.2 ASSAY OF SOD

SOD activity was estimated by the commercial Ransod kit (Randox Laboratories, UK). This method is based on the generation of $\cdot\text{O}_2$ produced by xanthine and xanthine oxidase (XOD), which react with phenyl tetrazolium chloride to form a red formazan dye. RBC-SOD activity was measured in RBCs haemolysate by the degree of inhibition of reaction. The results were expressed as U/ml. RBC-SOD by Winterbourns Method which is based on 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).

3.3 ASSAY OF CAT

CAT activity was measured by monitoring the decrease in absorption of H_2O_2 at 240 nm (19). 100 μl of the serum was added to a 0.5 ml quartz cuvette containing 400 μl of 20 mM H_2O_2 in PBS (25°C) and mixed thoroughly by pipetting. The absorbance was monitored immediately at 240 nm for three minute at an interval of one minute. Catalase activity was measured for each sample and the rate in mAU/ min/mg protein was averaged.

3.4 ASSAY OF GSH

GSH status analyses was assayed from blood samples obtained by 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 (MPA). To precipitate proteins completely, the samples were vortexed and incubated on ice for 10 ml. After centrifugation at 4°C at 12000 rpm for 10 min, the supernatants were filtered through a 0.2mm filter and diluted 5 times before being injected into the capillary electrophoresis system.

3.5 GPx ASSAY:

Glutathione peroxidase (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 mM NADPH, 0.25 mM H₂O₂, 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 $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$.

3.6 NITRIC OXIDE ASSAY

Serum was deproteinized first to convert nitric oxide to nitrate, the stable product of nitric oxide. The nitrate present in filtrate is then reduced to nitrite, which was measured by diazotization of sulphanilamide and coupling with naphthylethylene diamine as in Najawa & Cortas method (22).

3.7 iNOS ASSAY:

The inducible nitric oxide synthase activity was measured in vitro in blood lymphocytes (suspended in MEM @ 1×10^6 viable cells/ml) using arginine and Greiss reagent by the method of Stuehr and Marletta (23). Optical density of the citrulline formed was determined spectrophotometrically with a UV-VIS spectrophotometer (Shimazu) at 540 nm against control.

3.8 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 blue colour. Vitamin-A and E were measured by high performance liquid chromatography (HPLC) as per the modified method of Omu *et al.* (25). Briefly α -tocopherol acetate and retinol acetate were pipetted into an Eppendorf tube. To this, blood serum was added and vortexed; hexane extract of vitamin-A and E taken out in a glass tube, dried under nitrogen stream, and dissolved into methanol. Finally, this preparation was injected into a HPLC, fitted with reverse phase of C 18 column. The vitamins were eluted with methanol at a flow rate of 1.5ml /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 filters.

3.9 ASSAY OF TRACE ELEMENTS

Copper and zinc in serum were estimated by Atomic Absorption Spectrophotometer.

4. STATISTICS

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

5. RESULTS

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

The contents of GSH and activity of iNOS was increased significantly in the all in all groups of breast cancer patients as compared to control group ($p < 0.05$). The content of GSH was increased 23.52% in postoperative group as compared to preoperative group. The level of NO was decreased 24.32 % in post-operative group as compared to the pre-operative group, and activity of iNOS was also decreased 38.01% in post-operative group as compared to pre-operative group (Table 1).

Significantly decreased activity of RBC-SOD ($p < 0.05$) and levels of plasma vitamins-C, A and E ($p < 0.05$) were observed in all the cancer patients when compared with healthy controls. It was observed that the concomitant decline in the activity of RBC-SOD and levels of the vitamins were associated with the progression of cancer, but the levels of all vitamins were not significant in post-operative patients as compared

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Açıklamalı [p2]: Preoperative and postoperative terms are written in three different ways: preoperative, pre operative and pre-operative. It should be same within the whole text.

Açıklamalı [p3]: !

to pre-operative patients (Table 2). Cu/Zn ratio was also found to be significantly ($p < 0.05$) higher in the pre and post-operative patients when compared with healthy controls.

Açıklamalı [p4]: !

6. DISCUSSION

Increased oxidative stress and lipid peroxidation are implicated in carcinogenic processes. The magnitude of this damage depends not only on ROS levels but also on the body defense mechanisms against them mediated by various cellular antioxidants (8, 26).

MDA is produced by the oxidation of polyunsaturated fatty acids in membranes induced by free radicals, is an indicator of oxidative damage. Many studies have examined the possibility of a connection between lipid peroxidation and cancer (6,27). Higher plasma MDA levels have been reported in cancer patients than those in the controls (27). However, lower lipid peroxidation measured in plasma by thiobarbituric acid-reactive substances has also been reported in the breast cancer group compared to the control (28). In the present study, our findings are in agreement with most of the earlier studies which suggest that there might be some accumulation of ROS which cause significantly higher lipid peroxidation at cellular and molecular levels. ROS are derived from $\text{NO}\cdot$ and released from inflammatory cells. It can act on neighboring dividing epithelial cells, leading to somatic mutations in crucial cancer-causing genes (29). $\text{NO}\cdot$ produced by iNOS in solid tumors has been implicated in enhanced vascular permeability, 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 a deleterious compound, such as free radicals and hydroperoxides (31). GSH in the nucleus also maintains the redox state of critical protein sulphhydryls 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 the breast cancer patients support the hypothesis that the glutathione status is inversely related in malignant transformation (33). Several researches have reported decreases level of GSH in patients' blood with breast cancer compared to those of the control subjects (34,35). Our results

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showed that there were significant decreases in the levels GSH in blood of patient with breast cancer compared to those of 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.

The cell has strong endogenous antioxidant defenses against increased lipid peroxidation, ROS and NO. SOD and CAT are the first line of defense against superoxide and hydrogen peroxide (36, 37). The significant increase in SOD activities indicates the formation of more superoxide radicals and their removal as SOD metabolizes superoxide radicals (38). Furthermore the decrease in activity of SOD probable might be due to association with free radical generation which causes damage to enzyme by cross linking or damaging the nuclear DNA leading to mutations. It may also be due to scarcity of trace elements like zinc, manganese etc. which act as a cofactor for this enzyme (39). However, the significant decrease in CAT activity indicates the toxicity produced by H₂O₂ (27). Studies have shown that oxidants may activate gene expression through the antioxidant responsive elements (ARE) (40), which explains the enhanced enzyme activities. Our data have shown a significant increase in SOD and decrease in CAT activities in the patients with breast cancer than those of 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 antioxidant where it gets utilized. Its synergism with vitamin-E & A, helps in sparing of vitamin-E and during this process vitamin-C gets utilized (12, 13) which is seen as a significant decline in plasma ascorbic acid. Negative correlation ($r = -0.9.9.$) between vitamin-C and MDA was noted leading to the conclusion that free radicals are scavenged by ascorbic acid and thus it gets 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 $\cdot\text{OH}$, 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 integrity of the membrane. However cancerous cells may consume the zinc which is present in the circulation for tumor growth and to maintain its membrane integrity (43). This might be the possible reason of depletion of zinc in breast cancer. Increased ratio of Cu/Zn is due to the significant decrease in Zn and concomitant increase in copper. So in pre-operative group the ratio of Cu/Zn was increased as compared to control. As this ratio is altered, this could be considered as a risk factor for tumor growth or carcinogenesis.

In conclusion, breast cancer is related to 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 breast cancer patients before and after surgery. Prospective studies in a larger population should be carried out to demonstrate our present findings.

Açıklamalı [p7]: confirm OR support OR explain

7. ACKNOWLEDGEMENTS

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Key Words : Breast cancers, Oxidants, Antioxidants

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

Parameter s	Control	Pre-operative	Post-operative	% change of in post-operative group as compared to to pre-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, ↑ Increase, ↓ Decrease

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

	Control	Pre-operative	Post-operative	% change in post-operative group as compared to Pre-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	71.4 ± 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