Biochemistry

Alterations in Serum Antioxidant Profile In Patients with Metastatic Breast Cancer Undergoing Chemotherapy- An Observational Study

Charushila Y Kadam[1], Subodhini A Abhang[2*]

[1]Assistant Professor, Department of Biochemistry, RKDF Medical College, Hospital and Research Centre, Bhopal, Madhya Pradesh- 462026, India
[2]Professor, Department of Biochemistry, Symbiosis International (Deemed) University and Symbiosis Medical College for Woman, Pune, Maharashtra-411042, and India

*Corresponding author: Dr. Subodhini A Abhang

Abstract	Original Research Article

Background: The imbalance between pro- and antioxidant mechanisms favoring oxidative stress is known to be involved in pathogenesis of cancer. Antineoplastic agents used in treatment of metastatic breast cancer acts by generating reactive free radicals. Reactive radicals produced due to metastatic breast disease and metabolism of chemotherapy drugs are detoxified mainly through glutathione antioxidant system. *Objectives:* To evaluate differential serum glutathione peroxidase and glutathione-s-transferase activity and serum reduced glutathione concentrations among patients with metastatic breast disease receiving chemotherapy with different drugs. *Material and Methods:* We selected thirty metastatic female breast cancer patients and blood samples were collected before and after first chemotherapy cycle. Thirty healthy female volunteers from similar age group were selected as controls. Serum glutathione peroxidase activity was determined by ELISA. Serum glutathione-s-transferase activity and reduced glutathione concentration was measured by spectrophotometric methods. *Results:* Glutathione peroxidase and glutathione-s-transferase activity was significantly higher and serum reduced glutathione concentration was significantly lower in metastatic breast disease before receiving chemotherapy compare to healthy controls ($P<0.0001$). After chemotherapy, glutathione peroxidase ($P=0.022$), glutathione-s-transferase ($P<0.0001$) activity as well as reduced glutathione ($P<0.0001$) concentration were significantly decreased as compare to their concentration before chemotherapy. No significant difference was observed in the levels of these antioxidants among different chemotherapy groups. *Conclusion:* These findings provide an insight on systemic redox state and differential antioxidant status in metastatic breast disease among different chemotherapy treated groups. Evaluation of these antioxidants may be useful in chemotherapy treatment monitoring and prognosis of metastatic breast cancer.
Keywords: Antioxidants, Reduced glutathione, Glutathione-s-transferase, Glutathione peroxidase, Metastatic breast cancer, Chemotherapy.

INTRODUCTION

Breast cancer is the most frequent cancer in woman and is biologically most heterogeneous disease in its histology, molecular classification, and response to treatment and mortality rates [1, 2]. A remarkable proportion of breast cancer patients die due to development of metastasis. At metastatic stage, quality of life and patient survival depends on tumor sensitivity to anticancer drugs. In spite of the advances in approaches to treat metastatic breast cancer, chemotherapy is treatment of choice among these patients [2]. Chemotherapy drug efficacy is dose dependent. Anticancer drugs cause side-effects due to toxicity to non-tumor tissues [2, 3]. Chemotherapy drugs exert their cytotoxic effects essentially through redox cycling leading to reactive oxygen species and their byproduct formation causing cellular damage, specifically at the level of DNA [4, 5]. These adverse effects of free radicals causing cellular damage may cause gain of new mutations and development of treatment resistance in cancer [6]. Reactive oxygen species (ROS) cause tumor cell death either due to their direct cytotoxic effects or induction of intracellular apoptotic pathways [5]. The damage occurring in cancer cells depends on rate of formation of ROS and the effectiveness of cellular antioxidant mechanisms [4]. Therefore, various antioxidants and antioxidant enzymes influencing cellular ROS levels probably affect patient prognosis after chemotherapy treatment [5].

Several studies have demonstrated role of glutathione antioxidant system in different stages of carcinogenesis [5-11]. However, there is paucity of data on serum glutathione peroxidase (GPx) and glutathione-s-transferase (GST) activity and reduced glutathione (GSH) concentrations in patients with metastatic breast disease receiving different chemotherapy drugs. Therefore, in this study, we evaluated serum concentrations of these parameters in patients with metastatic breast cancer receiving first chemotherapy cycle with cytotoxic drugs and assessed differential antioxidant status among different treatment groups.

MATERIAL AND METHODS

In this prospective observational study, thirty female metastatic breast cancer patients receiving chemotherapy were included. The mean age of the patient was 55.26 ± 13.15 [Range 30-74 years]. The clinicopathological features of metastatic breast cancer patients are given in Table 1. Thirty healthy female volunteers from similar age group were included as controls. These female controls were not taking any regular medication or antioxidant supplements at the time of evaluation, were disease free, had no habit or prior history of smoking or drinking alcohol, and had no recent infections. This study was carried out from November 2010 to July 2014 in the Department of Biochemistry and study protocol was approved by institutional ethical committee.

Inclusion criteria

Thirty metastatic breast cancer patients were included in this study. The patients were treated with 5-Flurouracil+Epirubicin+Cyclophosphamide [FEC] or Adriamycin (Doxorubicin) +Cyclophosphamide [AC] or Paclitaxel [PC] (sequential) chemotherapy.

Exclusion criteria

Patients with allergic and infectious diseases, autoimmune diseases, systemic disorders, other malignancies and patients on antioxidant supplements were excluded to avoid false results.

Table-1: Clinicopathological features of thirty patients with metastatic breast disease

Parameter	Number
TNM stage	
Stage IV	30
Chemotherapy	
FEC	18
AC	7
PC	5

Sample Collection

5ml of venous blood samples were collected after informed written consent from controls and patients. Paired blood samples were collected from patients with metastatic breast cancer prior to chemotherapy and after three weeks of giving first cycle of FEC or AC or PC. The blood was centrifuged at 2000g for 10 minutes and separated serum was stored in aliquots for further analysis at -80°C. The chemicals required for spectrophotometric analysis were purchased from Alfa Aesar, South Korea. Glutathione peroxidase ELISA kit was purchased from Cayman Chemical Company, USA.

Estimation of serum GSH

GSH was measured by spectrophotometric method as described by Moron *et al*. [12]. Deprotenization was achieved by adding 3ml of 5% Trichloroacetic acid to 0.1 ml of serum. Contents of the tubes were mixed well and kept for 5 minutes before centrifugation. Then, 4 ml 0.3M Na_2HPO_4 having pH 8.0 and 0.5 ml 0.6mM DTNB was mixed to 1 ml of supernatant by vortexing and within 10 minutes, absorbance of yellow color produced was measured at 412 nm. Standard curve of glutathione was used to calculate serum GSH concentration and was expressed as mg/dl.

Estimation of serum GST activity

GST activity was measured by CDNB method [13]. For GST estimation, a reaction mixture was prepared by adding 850µl 0.1M phosphate buffer with pH 6.5 and 50 µl 20mM CDNB reagent. The reaction mixture was preincubated for 10 minutes at 37°C. The reaction was initiated by addition of 50µl 20mM GSH and 50µl serum in preincubated mixture. Reaction was then followed for 5 minutes by measuring absorbance for 1 minute interval at 340nm. Simultaneously, blank was prepared by using deionized water in place of serum. Using molar extinction coefficient ($9.6mM^{-1} cm^{-1}$) and change in absorbance/minute, GST activity was calculated.

Estimation of serum GPx activity

Serum glutathione peroxidase estimation was done by ELISA by using ELISA kit according to instructions of manufacturer [14]. Briefly, to background wells, 120 µl of assay buffer + 50 µl of co-substrate mixture were added. To positive control wells, 100 µl of assay buffer + 50µl of co-substrate mixture + 20 µl of GPx control were added. To sample wells, 100 µl of assay buffer + 50 µl of co-substrate mixture + 20 µl of serum were added. 20µl cumene hydroperoxide was added to each well to initiate the reaction. The contents of the plate were mixed by careful shaking for few seconds and absorbance was read using plate reader once every minute at 340nm to get at least five time points. The OD change/minute was determined (ΔA_{340}) and the reaction rate at 340nm was determined using the NADPH extinction coefficients of $0.00373µM^{-1}$, this value was adjusted for the 0.6 cm pathlength of the contents in each well. The serum GPx activity was expressed as nmol/min/ml.

Statistical Analysis

The data was collected and entered in excel sheet for analysis using MedCalc for Windows and SPSS version 17. It was expressed in terms of Mean ± Standard deviation for all parameters. Comparison of Mean and SD between controls and patients was done using unpaired t test and between patients before and after chemotherapy was done using paired t test to assess significance of mean difference between two groups. Results with P values less than 0.05 were considered as statistically significant.

RESULTS

In this study, statistically significantly higher serum GPx (Mean=82.44 ±34) and GST (Mean=14.25±4.13) activity was observed in metastatic breast cancer patients before chemotherapy as compare to enzyme activity in healthy controls (Mean=24.22±3.53 and Mean 1.81±1.21, respectively) (P<0.0001) [Figure 1 and Figure 2]. Statistically significantly lower serum GSH (Mean=2.29±0.78) concentration was observed in metastatic breast cancer patients before chemotherapy as compare to concentrations in healthy controls (Mean=3.96 ± 1.18)

(P<0.0001) [Figure 3]. A further significant decrease in serum GSH (Mean=1.76±0.55) concentration was observed in patients with metastatic disease after three weeks of receiving first chemotherapy cycle as compare to levels before chemotherapy (P<0.0001) [Figure 3]. A significantly decreased serum GST (Mean=6.41±1.16) (P<0.0001) and GPx (Mean=61.04±21.27) (P=0.022) activity was observed in patients with metastatic disease after three weeks of receiving first chemotherapy cycle as compare to levels before chemotherapy but serum activity of these enzymatic antioxidants was found significantly higher as compare to their activity in healthy controls (P<0.0001) [Figure 1 and Figure 2]. Further, serum GPx and GST activity and GSH concentration was compared among FEC, AC and Paclitaxel treated patients to identify the differential antioxidant status among three chemotherapy treatment groups. The mean serum levels of GPx, GST and GSH and their statistical comparison among three chemotherapy treatment groups is depicted in Table 2 and Table 3. Serum levels of these parameters were not statistically significantly different among three chemotherapy treatment groups in metastatic breast disease after three weeks of receiving first cycle.
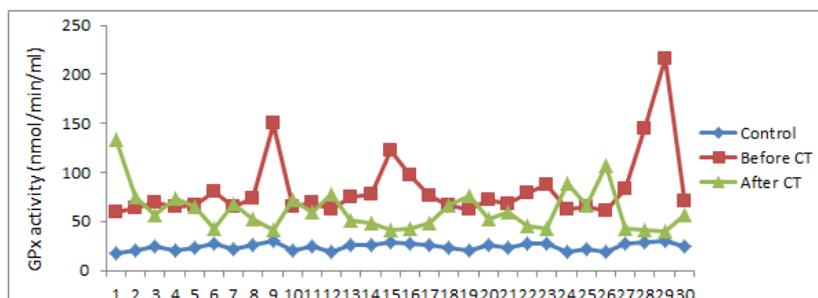

Fig-1: Serum GPx activity in controls and patients with metastatic breast cancer
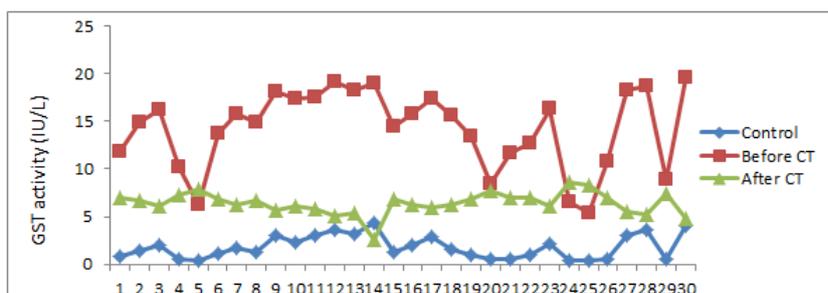

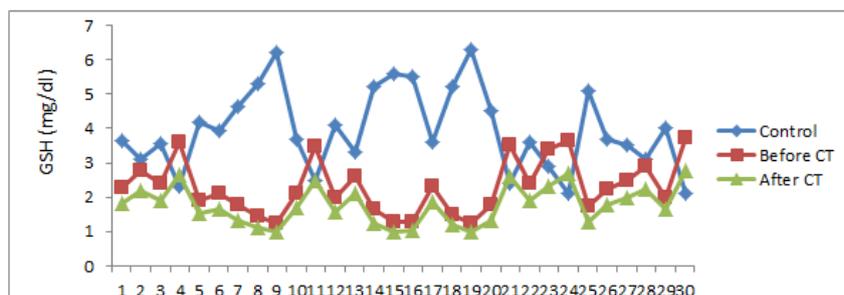**Fig-2: Serum GST activity in controls and patients with metastatic breast cancer**

Fig-3: Serum GSH concentration in controls and patients with metastatic breast cancer

Table-2: Serum levels of antioxidants among different treatment groups

Chemotherapy drugs	No. of patients	GSH (mg/dl)	GST (IU/L)	GPx (nmol/min/ml)
FEC	18	1.76 ± 0.60	6.51 ± 1.10	61.69 ±23.34
AC	07	1.64 ± 0.30	6.34 ± 0.53	54.09 ± 10.09
PC	05	1.94 ± 0.66	6.23 ± 2.14	68.47 ± 25.65

Values were expressed as Mean ± SD

Table-3: Statistical comparison of antioxidants among different treatment groups

Group	GSH	GST	GPx
FEC and AC	P=0.62 [a]	P=0.68 [a]	P=0.41 [a]
FEC and PC	P=0.57 [a]	P=0.68 [a]	P=0.57 [a]
AC and PC	P=0.31 [a]	P=0.90 [a]	P=0.20 [a]

[a] Statistically not significant

DISCUSSION

In the present study, effect of chemotherapy on serum GPx, GST and GSH was evaluated in patients with metastatic breast disease and also investigated a differential antioxidant status with respect to glutathione antioxidant system among paclitaxel and combination chemotherapy groups to assess the redox imbalance.

It is now established that formation of ROS is higher in rapidly proliferating cancer cells compare to normal diving cells. The presence of constitutive oxidative stress markers in breast carcinoma is confirmed by several reports [6, 7, 10, 15]. Cancerous cells at metastatic stage are under substantial oxidative stress because of increased metabolic activities and require potent antioxidant system to maintain viability [16]. To prevent oxidative stress mediated cytotoxic effects and to maintain proliferative potential and the capacity for tumor initiation, cancer stem cells require an increased antioxidant status [16]. Downregulation and genetic imbalance among glutathione peroxidases were found to play a significant role in breast cancer [9]. In the present study, we reported significantly higher serum GPx (P<0.0001) and GST (P<0.0001) activity and significantly lower serum GSH concentration in patients with metastatic breast cancer before chemotherapy as compare to healthy controls. Howie AF *et al*. [11] reported significantly higher expression of GPx and GST in breast cancer tissue compare to normal tissue and correlates with our findings. However, in contrast to our findings, Kangari P *et al*. [7] reported a decreased circulating glutathione peroxidase activity in patients from different pathological stages of breast cancer. Mehdi *et al.* [17] reported decreased reduced glutathione in serum as well as cancerous tissue in patients with breast carcinoma as compare to control group. Our findings of decreased serum GSH among patients with metastatic breast disease compare to control group correlated well with their reports. The observed decrease in serum reduced glutathione indicates higher oxidative stress in metastatic disease that causes oxidation of reduced glutathione [17]. Redox imbalance caused by increased production of ROS was more pronounced in metastatic breast cancer patients. This was indicated by depletion of reduced glutathione levels and as an adaptive mechanism, glutathione peroxidase and glutathione-s-transferase was elevated [17, 18]. An immunohistochemical analysis of invasive ductal carcinoma revealed that high glutathione peroxidase 1 expression is linked with increased mortality and decreased survival in breast cancer subtype [19]. After administration of first chemotherapy cycle, significantly decreased serum GPx and GST activity and GSH levels were observed as compare to levels before chemotherapy. However, serum levels of GPx and GST were still significantly higher as compare to healthy controls. Further, serum levels of these antioxidants among different chemotherapy treatment groups were compared. No significant difference in the levels of these antioxidants among three chemotherapy groups was observed. Many classes of chemotherapeutic drugs are designed to elevate cellular oxidative stress with the goal to bring about irreparable damages subsequently resulting in tumor cell apoptosis [3]. ROS induces death of tumor cells either due to their direct cytotoxic effect or by triggering intracellular apoptotic pathways [5]. Junior ALG *et al*. [20] reported decreased serum GSH and GPx in breast cancer patients after receiving second and forth chemotherapy cycle with adriamycin and cyclophosphamide compare to baseline levels. Braganca SF *et al*. [21] reported a significant decrease in serum glutathione peroxidase among breast cancer patients treated with Adriamycin, cyclophosphamide or Adriamycin, cyclophosphamide and paclitaxel or docetaxel, Adriamycin and cyclophosphamide after 12 months of receiving chemotherapy compared to levels at six months. Kasapovic J *et al*. [22] reported decreased serum reduced glutathione and glutathione peroxidase in response to 5-fluorouracil, doxorubicin and cyclophosphamide in patients with breast cancer. Our findings of decreased reduced glutathione and glutathione peroxidase after first chemotherapy cycle of Adriamycin, cyclophosphamide (AC) and 5-fluorouracil, epirubicin, cyclophosphamide (FEC) are in line with their reports. Jardim *et al*. [10] reported high glutathione peroxidase expression was associated with shorter survival in breast cancer patients receiving adjuvant chemotherapy. Han *et al*. [23] reported an increased sensitivity to doxorubicin in MCF-7 breast

cancer cell line when levels of reduced glutathione decreased. A higher expression of glutathione-s-transferase combined with high reduced glutathione can increase the rate of detoxification by conjugation of chemotherapeutic agents and reduces their effectiveness [24]. Erat M, *et al.* [25] showed that paclitaxel and cyclophosphamide non-competitively inhibits the in vitro enzyme activity of glutathione-s-transferase from human erythrocytes. The observed decrease in serum GPx, GST activity and GSH concentration in breast cancer among chemotherapy treated groups might be due to role of these antioxidants in conjugation and detoxification of antineoplastic drugs, their metabolites and/or due to inhibition of enzyme activities by antineoplastic drugs [22, 25]. Depletion of these antioxidants after chemotherapy facilitated oxidative shift and potentiated already existing chronic oxidative stress associated with metastatic breast cancer. In this study, chemotherapy induced early and comparative changes in glutathione antioxidant system in metastatic breast cancer patients were reported for the first time among three treatment groups with sequential paclitaxel or combination of drugs. However, relatively small numbers of cases were included in different chemotherapy groups. Furthermore, as the effect of one chemotherapy cycle was studied, an association between glutathione antioxidant system and patients' response to chemotherapy could not be determined. Therefore, our report should be considered as preliminary findings and more studies with different treatment arms and further follow-up are required to establish a prognostic significance of these antioxidants and response to chemotherapy in metastatic breast cancer.

CONCLUSION

In conclusion, the results of the study suggests redox imbalance in metastatic breast cancer as indicated by decreased serum GSH concentration and higher serum GPx and GST activity as an adaptive mechanism to circumvent increased ROS. After first chemotherapy cycle, serum GPx, GST activity and GSH concentration were decreased significantly. Further, these serum antioxidants were compared among FEC, AC and PC treated groups. No statistically significant difference was observed among these antioxidants in different chemotherapy treated groups. Depletion of these antioxidants after chemotherapy promoted further oxidative shift and potentiated oxidative stress linked to metastatic disease. Evaluation of these antioxidants may be useful in chemotherapy treatment monitoring.

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