

Evaluation of Serum Antioxidants during Adjuvant Chemotherapy of Breast Cancer- A Prospective Observational Study

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Abstract

Background: The enzymatic and non-enzymatic antioxidant defense systems of the cell prevent oxidant mediated damage to different biomolecules by neutralizing free radicals generated during anticancer drug metabolism. Reduced glutathione (GSH) and glutathione dependent enzymes such as glutathione peroxidase (GP_x) and glutathione-s-transferase (GST) are the key determinants of cellular response to oxidative stress. The objective of this study was to evaluate changes in serum levels of these antioxidants after first cycle of adjuvant chemotherapy in breast cancer patients.

Material and Methods: Histopathologically proven 60 breast cancer patients were included in the present study. The blood samples were taken after surgery before chemotherapy and after 3 weeks of administration of first cycle of chemotherapy. 30 healthy female controls were selected for comparison. Serum GSH and GST were estimated by spectrophotometric methods and serum GP_x was estimated by ELISA.

Results: The serum levels of GSH, GST and GP_x were significantly decreased ($P < 0.0001$) after first cycle of adjuvant chemotherapy in breast cancer patients as compare to levels before chemotherapy. A significant positive correlation was found between serum levels of GSH and GST as well as GSH and GP_x after first chemotherapy cycle.

Conclusion: Our data suggests that administration of chemotherapeutic drugs causes depletion of reduced glutathione and glutathione-dependent enzymatic antioxidants such as GST and GP_x, which may be due to higher oxidative stress after chemotherapy.

Keywords:

Breast cancer; Adjuvant chemotherapy; Reduced glutathione; Glutathione-s-transferase; Glutathione peroxidase; Antioxidants

Introduction

Breast cancer is one of the most common types of cancer in women and is second leading cause of cancer-related deaths in women worldwide [1,2]. The chance of developing invasive breast cancer at some time in women's life is a little less than 1 in 8. Breast cancer accounts for 25% to 32% of all female cancers in India. According to most recent estimates of IARC (International Agency for Research on Cancer, WHO), about 44,937 new cases of invasive breast cancer were diagnosed and about 70,218 women were died due to the disease in India [3,4].

The etiology of breast cancer is multifactorial. Significant risk factors includes early age at menarche, late age at menopause, family history, use of oral contraceptives, mutations in BRCA 1 and BRCA 2 etc [5,6]. The main presenting features in women with symptomatic breast cancer include a lump in the breast, nipple change or discharge and skin contour changes. Definitive diagnosis is done by biopsy and histopathology. The treatment of choice for breast cancer is surgical intervention with chemotherapy, radiotherapy and anti-hormonal

therapy either in the neoadjuvant and/or adjuvant setting depending on stage of disease [7].

Adjuvant chemotherapy is a standard systemic treatment for invasive breast cancer after surgical removal of tumor [2]. Cytotoxic chemotherapy drugs kill cancer cells predominantly by triggering apoptosis in them. Several studies have shown that antineoplastic drugs used in treatment of cancer eliminate tumor cells by inducing apoptosis in them and this, in part at least, is done by generating reactive oxygen species in them [8-10].

The enzymatic and non-enzymatic antioxidant defense systems of the cell prevent oxidant mediated damage to different biomolecules such as lipids, protein and DNA by neutralizing free radicals [11,12]. However, these antioxidants may inhibit apoptosis in cancerous cells induced by oxidative stress following chemotherapy by scavenging free radicals and may exert antiapoptotic and cancer promoting effects in cancer patients [13,14]. Reduced glutathione (GSH) and glutathione dependent enzymes such as glutathione peroxidase (GP_x) and glutathione-s-transferase (GST) are the key determinants of cellular response to oxidative stress [15]. Glutathione (GSH) is ubiquitous,

most abundant, low molecular weight thiol-containing tripeptide in cells [16]. GSH effectively scavenges free radicals and other reactive oxygen species (e.g. hydroxyl radical, peroxy nitrite and H₂O₂) directly and indirectly through enzymatic reactions [17]. In addition, GSH is involved in other biological functions including its role in maintaining body homeostasis and protecting cells against toxicants, DNA-damaging agents and carcinogens of either exogenous or endogenous source [18]. Glutathione-s-transferases (GSTs) are an enzyme superfamily involved in the phase-II metabolism. GSTs catalyze the conjugation of glutathione to electrophilic species resulting in hydrophilic species that are more readily excreted. GSTs play an important role in drug metabolism including many cancer therapeutic agents [19]. It has been known that GSTs can reduce lipid hydroperoxides through their Se-independent glutathione peroxidase activity and that these enzymes can also detoxify lipid peroxidation end products such as 4-hydroxynonenal [20]. GST has recently been implicated in protection against apoptosis [14]. Glutathione peroxidase (GPx) is a key enzyme responsible for detoxification of cellular hydrogen peroxide using GSH; it exists in two forms- selenium dependent and selenium independent. The selenium-dependent GP_x both detoxifies H₂O₂ and converts lipid hydroperoxides to non-toxic alcohols, whereas the selenium-independent enzyme is responsible only for metabolizing lipid peroxides [21-23].

The measurement of reduced glutathione in tissues and body fluids is used as an index of the oxidative stress that occurs under different pathological conditions e.g. cancer [24]. Several studies have shown that the serum levels of GSH were decreased in patients with prostate cancer and breast cancer as compared to controls which may be due to the response of increased reactive oxygen metabolite production [24-27]. However, some studies have reported increased serum levels of GSH in breast cancer patients as compared to controls [6]. In addition to this, GSTs have been reported to accumulate in various human cancer tissues [28-30]. The GST (Pi) is present at high levels in many solid tumors particularly ovarian, small cell lung, breast, liver, oesophagus, stomach, colon and pancreas [28-30]. The elevated serum levels of GST have been reported previously in breast, prostate, colon and oesophagus cancer [6,26,30]. However, some reports have shown decreased levels of GST in patients with breast cancer [31-33]. Similarly, the higher levels of GP_x have been reported in patients with benign and malignant breast cancer as well as in patients with transitional cell carcinoma bladder [6,15,22-24,34]. However, some studies have reported decreased levels of GP_x in breast, gastric, colorectal and prostate cancer [26,35]. Thus, available data on serum levels of GSH, GP_x as well as GST is contradictory. Furthermore, the serum levels of these antioxidants are very less studied in breast cancer patients before and after adjuvant chemotherapy.

Parameter	Number
Age	
Range	30-75 years
Diagnosis	
Invasive ductal carcinoma	47
Invasive lobular carcinoma	13
Pathological TNM stage	
Stage II	30
Stage III	30
Receptor status	
ER +	46
PR +	41
HER2/neu +	36
Menopausal status	
Premenopausal	21
Postmenopausal	39
Surgery	
Breast conserving surgery (BCS)	19
Modified radical mastectomy (MRM)	41

Table 1: Clinicopathologic characteristics of 60 breast cancer patients.

Therefore, considering paucity in the studies on serum levels of these antioxidants in breast cancer patients after adjuvant

chemotherapy, we evaluated their levels in serum before and after adjuvant chemotherapy.

Material and Methods

The present study was prospective observational study. Histopathologically proven 60 female breast cancer patients with invasive ductal/lobular carcinoma with stage II (30 patients) and stage III (30 patients) as classified TNM system were included in the present study. The patients were aged between 30 and 75 years. 30 healthy and age matched female controls were selected for comparison. Clinicopathological characteristics of patients are given in Table 1.

The study was approved by institutional ethical committee (Reference number: BJMC/IEC/Pharmac/D 1210137-39). After obtaining prior written consent, 5 ml of venous blood was drawn under aseptic precaution after surgery before chemotherapy and 3 weeks after administration of first cycle of 5-fluorouracil, epirubicin, cyclophosphamide (FEC)/Adriamycin, cyclophosphamide (AC)/paclitaxel (standard dose) chemotherapy. The serum was separated and stored at -80°C until analysis.

To avoid false positive results, care was taken to exclude patients with systemic disorders, infectious diseases, allergic diseases, autoimmune diseases, other malignancies and radiation treated patients.

The required chemicals- reduced glutathione, 1-chloro-4-dinitrobenzene (CDNB) and 5-5'-Dithiobis, 2-nitrobenzoic acid were purchased from Alfa Aesar, South Korea. The serum level of GP_x was determined by ELISA using ELISA kit (Cayman chemical company, USA).

Measurement of reduced glutathione (GSH)

Serum reduced glutathione was measured by method of Moron et al [36]. 0.1 ml of serum was deproteinized by 3 ml of 5% TCA. After mixing, tubes were kept for 5 min at room temperature and then centrifuged. To 1 ml of supernatant 4 ml of 0.3M Na₂HPO₄ (pH: 8.0) and 0.5 ml of 0.6 mM DTNB was added. The contents were mixed by vortexing and absorbance of yellow color produced was recorded within 10 min at 412 nm. The concentration of GSH from serum was calculated by use of standard curve of GSH. The values were expressed as mg/dl.

Measurement of glutathione-s-transferase (GST)

Serum GST was estimated by CDNB method [37]. GST was estimated in 1 ml of incubation mixture containing 850 µl of 0.1M phosphate buffer of pH 6.5 and 50 µl of 20 mM CDNB reagent, preincubated at 37°C for 10 min. Reaction was started by adding 50 µl of 20 mM GSH and 50 µl of serum. Reaction was followed at 1 min interval for 5 min by measuring absorption at 340 nm. The blank was run by adding deionized water instead of serum. Then change in OD/min was calculated. Estimation of GST was done by using the molar extinction coefficient (9.6 mM⁻¹cm⁻¹) of GST. GST values were expressed as IU/L.

Measurement of glutathione peroxidase (GP_x)

The measurement of serum glutathione peroxidase was done by ELISA according to instructions of manufacturer (Cayman Chemical Company, USA) [38]. Briefly, 100 µl of assay buffer, 50 µl of co-substrate mixture and 20 µl of sample was added to sample wells. For non-enzymatic well 20 µl of distilled water and for control well 20 µl of GP_x control were added instead of serum sample. The reaction was

started by adding 20 µl of cumene hydroperoxide to all the wells being used. Plate was shaken carefully for few seconds to mix. The absorbance was read once every minute at 340 nm using a plate reader to obtain at least 5 time points. The change in absorbance per minute was determined (ΔA_{340}). The GP_x values were expressed in terms of nmol/min/ml. (CV: intra-assay 5.7%, inter-assay 7.2%).

Statistical analysis

The data for biochemical analysis was expressed as Mean±SD. The statistical significance of the results was analyzed by using one way ANOVA and student's t test. Value of P<0.05 was considered statistically significant. Bivariate correlation analysis was used for determining correlations between measured parameters before and after chemotherapy.

Results

Table 2 shows the mean serum levels of reduced glutathione, glutathione-s-transferase and glutathione peroxidase in healthy controls and stage II as well as stage III breast cancer patients before and after adjuvant chemotherapy (FEC/AC/PC). The serum levels of reduced glutathione were significantly lower in post-operative breast cancer patients before chemotherapy in stage II (P=0.0004) as well as stage III (P<0.0001) of the disease as compare to levels in healthy controls. Further significant decrease in the levels of GSH was observed in stage II as well as stage III breast cancer patients after 3 weeks of receiving first cycle of adjuvant chemotherapy as compare to levels before chemotherapy (P<0.0001) and levels in healthy controls (P<0.0001). The serum levels of glutathione-s-transferase and glutathione peroxidase were significantly higher in post-operative stage II (P<0.0001) as well as stage III (P<0.0001) breast cancer patients before chemotherapy as compare to levels in healthy controls. After 3 weeks of administration of first cycle of chemotherapy, we found a significant decrease in the serum levels of glutathione-s-transferase and glutathione peroxidase in stage II as well as stage III of the disease as compare to levels before chemotherapy (P<0.0001) but the values were still significantly higher as compare to levels in healthy controls (P<0.0001). A significant inverse correlation was observed between serum GSH and GST as well as serum GSH and GP_x in post-operative breast cancer patients before chemotherapy. However, a significant positive correlation was found between serum GSH and GST as well as serum GSH and GP_x after first cycle of chemotherapy (Table 3).

Discussion

In the present study, we evaluated the serum levels of antioxidants such as reduced glutathione, glutathione-s-transferase and glutathione peroxidase in post-operative breast cancer patients undergoing first cycle of adjuvant chemotherapy (FEC/AC/PC).

Cytotoxic drugs have been known to produce highly reactive free radicals that act as common mediators of apoptosis during chemotherapy treatment of cancer [8-10,39]. The oxidative stress is the condition of overproduction of reactive free radicals or the depletion of antioxidants [40]. The generation of free radicals is controlled by large number of antioxidant systems of body. Reduced glutathione is one of the principle intracellular antioxidants. The glutathione and glutathione dependent enzymes directly scavenges free radicals and protects cells from oxidative insults. However, this

may cause apoptosis resistance in cancerous cells following chemotherapy [11].

Subjects	No. of cases	GSH (mg/dl)	GST (IU/L)	GPx (nmol/min/ml)
Healthy controls	30	3.96 ± 1.18	1.81 ± 1.21	24.22 ± 3.53
Breast cancer patients				
Stage II before chemotherapy	30	2.84 ± 0.42e	12.58 ± 3.90a	51.79 ± 8.55a
Stage II after first cycle of chemotherapy	30	1.89 ± 0.40ab	5.33 ± 1.54ab	36.53 ± 5.62ab
Stage III before chemotherapy	30	2.33 ± 0.82ab	13.01 ± 3.79ab	69.11 ± 16.50ab
Stage III after first cycle chemotherapy	30	1.79 ± 0.53ac	5.96 ± 1.38ac	50.31 ± 13.07ac
The data was expressed as Mean ± SD. a P<0.0001 Significant when compared to healthy controls. e P=0.0004 Significant when compared to healthy controls. b P<0.0001 Significant when compared to stage II breast cancer patients before chemotherapy. c P<0.0001 Significant when compared to stage III breast cancer patients before chemotherapy.				

Table 2: Depict serum levels of reduced glutathione (GSH), glutathione-s-transferase (GST) and glutathione peroxidase (GPx) in healthy controls and post-operative breast cancer patients before and after chemotherapy.

Parameters	After first chemotherapy cycle in stage II of disease.	After first chemotherapy cycle in stage III of disease.
GSH/GST	r= +0.86	r= +0.89
GSH/GPx	r= +0.89	r= +0.94

Table 3: Correlation analysis of measured parameters after first chemotherapy cycle.

In the present study, we have observed significantly lower levels of reduced glutathione in post-operative breast cancer patients before chemotherapy in stage II (P=0.0004) as well as stage III (P<0.0001) of the disease as compare to levels in healthy controls. The decreased level of reduced glutathione after surgery has been reported in patients with gastric cancer by Czczot et al. [41] as well as in patients with breast cancer (stage II) in our previous study [2]. However, in contrast to our finding, elevated level of GSH in breast cancer patients and non-significant decrease in the levels after 3 weeks of mastectomy have also been reported [6,42]. The depletion of reduced glutathione is an index of oxidative stress. The higher level of nitric oxide, which may increase production of peroxynitrite and, further oxidation of GSH or increased utilization of GSH for detoxification of lipid hydroperoxides, formed due to high oxidative stress in post-operative breast cancer patients, as described in our previous report [2], might be responsible for this depletion of GSH. Further, significant decrease in the serum level of GSH was observed in stage II as well as stage III of disease after first cycle of chemotherapy as compare to levels before chemotherapy (P<0.0001) and levels in healthy controls (P<0.0001). Our finding is in agreement with previous study reports [8,10]. The metabolism of antineoplastic drugs produces highly reactive electrophiles and decrease in the levels of GSH after chemotherapy indicates aggravation of oxidative stress which was possibly due to electrophilic burden on cells [13].

Reduced glutathione besides its role as scavenger of free radicals also acts in association with the detoxification enzymes glutathione peroxidase (GP_x) and glutathione-s-transferase (GST). These enzymes

protect cell from noxious substances by catalyzing conjugation reactions with reduced glutathione and prevents damage caused by reactive oxygen species by reducing hydrogen peroxide, lipid and phospholipid hydroperoxides [14,23]. This antioxidant function of reduced glutathione along with its associated enzymes however, may provide protection against oxidative stress mediated apoptosis [43]. The levels of GST and GP_x in serum were found to be elevated in most of the human cancers studied [23,37,43]. The post-operative higher values of GST were reported in patients with breast cancer and cancer of digestive tract [44,45]. In this study, we found significantly higher values of serum GST and GP_x in stage II (P<0.0001) as well as stage III (P<0.0001) post-operative breast cancer patients before chemotherapy as compare to levels in healthy controls. Our finding is in accordance with these previous study reports [44,45]. The higher values were observed post-operatively might be because normalization of GST [44] and GP_x levels after surgery may take about a month. Further, we found significant decrease in the serum levels of GST as well as GP_x after first cycle of adjuvant chemotherapy in stage II (P<0.0001) as well as stage III (P<0.0001) of the disease as compare to levels before chemotherapy, however, levels of both these enzymes were significantly higher in stage II (P<0.0001) as well as stage III (P<0.0001) breast cancer patients as compare to levels in healthy controls. Similar findings were reported by Chakraborty et al. [13]. A significant positive correlation was observed between serum levels of GSH and GST as well as GSH and GP_x after first cycle of chemotherapy. GST and GP_x may utilize GSH in detoxification of highly reactive electrophiles produced during cytotoxic action of chemotherapy drugs [13]. The decreased levels of both these enzymes

observed in the present study may be related with decrease in GSH after chemotherapy.

Conclusion

Our data suggests that administration of chemotherapeutic drugs (FEC/AC/PC) causes depletion of serum reduced glutathione and glutathione-dependent enzymatic antioxidants such as GST and GP_x, which may be due to higher oxidative stress after chemotherapy. However, the present study reports the effect of first chemotherapy cycle. Further follow-up with large study group is needed to establish the association between glutathione associated antioxidants and cancer chemotherapy.

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