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Correlation of Serum levels of Reduced Glutathione and Glutathione dependent Enzymes with Cytochrome c after 1st cycle of adjuvant Chemotherapy in Breast Cancer.

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Abstract

Several studies have shown that cytotoxic drugs used in chemotherapy causes apoptosis induction in cancerous cells by producing reactive oxygen species. The antioxidants neutralize these highly reactive free radicals and may confer resistance to chemotherapy induced apoptosis. There is very limited data on the serum antioxidants and their relationship with apoptosis markers in breast cancer patients undergoing chemotherapy. To measure serum levels of cytochrome c and antioxidants such as reduced glutathione (GSH), glutathione-s-transferase (GST) and glutathione peroxidase (GPx) and to find out correlation between them in breast cancer patients undergoing adjuvant chemotherapy. Histopathologically confirmed 60 breast cancer patients were included in the present study. 30 healthy controls were selected for comparison. Blood samples were collected from healthy controls and post-operative breast cancer patients before and after chemotherapy. Serum reduced glutathione and glutathione-s-transferase were measured by spectrophotometric methods and serum glutathione peroxidase and cytochrome c by ELISA. The serum levels of reduced glutathione, glutathione-s-transferase and glutathione peroxidase were significantly decreased (P<0.0001) where as serum level of cytochrome c was significantly increased (P<0.0001) after chemotherapy in breast cancer patients as compare to levels before chemotherapy. A significant inverse correlation was found between serum levels of these antioxidants and cytochrome c after chemotherapy. Our data suggests that, administration of chemotherapeutic drugs causes decreased levels of antioxidants such as GSH, GST and GPx, which may be due to counterbalance of oxidative stress. The increased level of apoptotic marker, cytochrome c might be associated with depletion of these antioxidants after chemotherapy. The elevated serum cytochrome c after chemotherapy indicates therapy induced cell death burden and evaluation of its level may help clinicians to predict effectiveness of chemotherapy.

Keywords: Cytochrome c, reduced glutathione, glutathione-s-transferase, glutathione peroxidase, apoptosis, adjuvant chemotherapy.

Introduction

Breast carcinoma is the most common type of neoplasm and is a leading cause of cancer related deaths in women worldwide[1,2]. Though breast cancer can be detected at early stage, death occurs in some patients due to metastasis and recurrence. Chemotherapy in neoadjuvant or adjuvant setting is one of the mainstays of medical intervention for breast cancer [2]. Several clinical and experimental studies have shown that cytotoxic drugs used in chemotherapy for breast cancer kill tumor cells by inducing apoptosis in them and this, in part at least, is done by generating highly reactive free radicals and reactive oxygen species[3-5]. The enzymatic and non-enzymatic antioxidant defense systems of the cell prevent oxidant mediated damage to different biomolecules such as lipids, proteins and DNA by neutralizing these free radicals. Thus antioxidant plays a protective role in healthy individuals against oxidative insults[6,7]. Reduced glutathione and glutathione dependent enzymes such as glutathione peroxidase and glutathione-s-transferase are the key determinants of cellular response to oxidative stress[8]. Reduced glutathione (GSH) is the major thiol in cells. It acts as an important antioxidant and, in conjunction with glutathione-s-transferase (GST) and glutathione peroxidase (GPx) plays central role in defense against free radicals and lipid hydroperoxides[8,9]. Glutathione-s-transferase is multifunctional enzyme which along with GSH plays an important role in detoxification of reactive radicals generated during anticancer drug metabolism[10,11]. However, these same 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[6,12,13]. One critical step in the apoptosis cascade is the cytochrome c release from mitochondria. Recently, it has been found that antioxidants acts quite upstream in the apoptosis cascade and inhibits the apoptosis induced by oxidative stress by blocking the release of cytochrome from mitochondria[4]. The altered serum levels of antioxidants like reduced glutathione, glutathione peroxidase and glutathione-s-transferase have been determined previously in breast cancer patients [1, 8- 11]. However, there is very limited data on the serum levels of these

antioxidants in breast cancer patients undergoing adjuvant chemotherapy. The relationship of these antioxidants with apoptosis marker such as cytochrome c following adjuvant chemotherapy in breast cancer patients is still not described.

Therefore, the present study was undertaken to evaluate the changes in serum levels of antioxidants such as reduced glutathione, glutathione-s-transferase and glutathione peroxidase and to assess their relationship with cytochrome c, an apoptosis marker, after 1st adjuvant chemotherapy cycle in breast cancer patients.

Material and Methods

The present study was carried out in B. J. Medical College and Sassoon General Hospital, Pune, Maharashtra, India. Clinically and histopathologically proven 60 female breast cancer patients diagnosed with invasive ductal/lobular carcinoma and with no history of alcohol or smoking were included in the present study. 30 healthy and age matched female controls were selected for comparison. To avoid false positive results, patients with infectious diseases, allergic diseases, hepatic and cardiac disorders, autoimmune diseases, diabetes mellitus and systemic diseases were excluded from the study. Patient's clinicopathologic characteristics are listed in table-1

Table-1
Clinicopathologic characteristics of 60 breast cancer patients.

Parameter	Number
Age	
Range	30-75 years
Mean	52.5 ± 13.42
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 mastcetomy (MRM)	41

The study was approved by Institutional Ethical Committee (Ref. No. BJMC / IEC / Pharmac / D 1210137-39). Prior written consent was taken from each healthy volunteer and breast cancer patient. Blood samples were collected from healthy female controls. From post-operative breast cancer patients, blood was collected before start of chemotherapy and after 3 weeks of receiving 1st adjuvant cycle of 5-fluorouracil, epirubicin, cyclophosphamide (FEC)/ Adriamycin (doxorubicin), cyclophosphamide (AC)/ paclitaxel. The serum was separated by centrifugation and stored in aliquots at -80ºC until analysis.

The required chemicals- reduced glutathione, 1-chloro-2,4-dinitrobenzene (CDNB) and 5-5'-dithiobis, 2-nitrobenzoic acid (DTNB) were purchased from Alfa Aesar, South Korea. The ELISA kit for cytochrome c estimation was purchased from Diaclone SAS, France and ELISA kit for glutathione peroxidase estimation was purchased from Cayman Chemical Company, USA.

Measurement of reduced glutathione (GSH): Serum reduced glutathione was measured by method of Moron et al [14]. 0.1 ml of serum was deproteinized by 3ml 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 Na_2HPO_4 (pH: 8.0) and 0.5ml of 0.6mM DTNB was added. The contents were mixed by vortexing and absorbance of yellow color produced was recorded within 10 min at 412nm. 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[15]. GST was estimated in 1ml of incubation mixture containing 850µl of 0.1M phosphate buffer of pH 6.5 and 50µl of 20mM CDNB reagent, preincubated at 37ºC for 10 min. Reaction was started by adding 50µl of 20mM GSH and 50µl of serum. Reaction was followed at 1min interval for 5 min by measuring absorption at 340nm. 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 (9.6mM $^{-1}cm^{-1}$) of GST. GST values were expressed as IU/L.

Measurement of glutathione peroxidase (GPx): The measurement of serum glutathione peroxidase was done by ELISA according to instructions of manufacturer (Cayman Chemical Company, USA) [16]. Briefly, 100µl of assay buffer, 50µl of co-substrate mixture and 20 µl of sample was added to sample wells. For non-enzymatic well 20µl of distilled water and for control well 20µl of GPx control were added instead of serum sample. The reaction was started by adding 20µl 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 340nm using a plate reader to obtain at least 5 time points. The change in absorbance per minute was determined (ΔA_{340}). The GPx values were expressed in terms of nmol/min/ml. [CV: intra-assay 5.7%, inter-assay 7.2%].

Measurement of cytochrome c (Cyt c): The measurement of serum cytochrome c was done by sandwich ELISA according to instructions of manufacturer (Diaclone SAS, France) [17]. Briefly,

pre-coated plate was removed from the sealed pouch and it was washed 3 times with wash buffer. 100µl of each standard, zero and 100µl of sample was added to appropriate number of wells. 50µl of diluted biotinylated conjugate was added to all wells and plates were incubated at room temp for 2 hours. The plate was washed for 3 times with wash buffer then 100µl of Streptavidin-HRP solution was added into all wells and incubated plate at room temperature (18-25ºC) for 1 hour. Wash step was repeated and 100µl of ready-to-use TMB substrate solution was added into all wells. Plate was incubated in dark for 10 minutes at room temperature and 100µl of stop reagent was added into all wells. The absorbance value of each well was read (immediately) on a spectrophotometer using 450nm as the primary wavelength and optionally 630nm as the reference wavelength. The standard curve was prepared for concentrations 10ng/ml to 0.3125ng/ml. The concentrations of cytochrome c from sample wells were determined by extrapolating OD values against cytochrome c standard concentrations using the standard curve [CV: intra-assay 6% and inter-assay 3.16%].

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. The strength of association between the measured parameters was determined by the correlation coefficient analysis.

Results and Discussion

Table-2 and table-3 shows the mean serum levels of reduced glutathione, glutathione-s-transferase, glutathione peroxidase and cytochrome c in healthy controls and stage II as well as stage III breast cancer patients before and after adjuvant chemotherapy. 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 1st 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, glutathione peroxidase and cytochrome c 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 1st 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). The serum level of cytochrome c was found significantly increased in stage II as well as stage III of the disease after 3 weeks of administration of 1st cycle of chemotherapy as compare to levels before chemotherapy (P<0.0001) and levels in healthy controls (P< 0.0001). A significant positive correlation was found between serum GSH and GST as well as serum GSH and GPx after 1st cycle of chemotherapy. A significant inverse correlation was found between serum levels of GSH and cytochrome c, GST and cytochrome c as well as GPx and cytochrome c after 1st cycle of chemotherapy (table-4).

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.

Discussion: In the present study, we have evaluated the association between serum antioxidants such as reduced glutathione, glutathione-s-transferase and glutathione peroxidase, with an apoptosis marker, cytochrome c during adjuvant chemotherapy in breast cancer patients.

Cytotoxic drugs have been known to produce highly reactive free radicals that act as common mediators of apoptosis during chemotherapy treatment of cancer[3-5,18]. The oxidative stress is the condition of overproduction of reactive free radicals or the depletion of antioxidants[19]. 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.

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

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±0.42[e]	12.58±3.90[a]	51.79±8.55[a]
Stage II after 1st cycle of chemotherapy	30	1.89±0.40[ab]	5.33±1.54[ab]	36.53±5.62[ab]
Stage III before chemotherapy	30	2.33±0.82[ab]	13.01±3.79[ab]	69.11±16.50[ab]
Stage III after 1st cycle chemotherapy	30	1.79±0.53[ac]	5.96±1.38[ac]	50.31±13.07[ac]

Table-3
Depict serum levels of cytochrome c in healthy controls and post-operative breast cancer patients before and after chemotherapy

Subjects	No. of cases	Cytochrome c (pg/ml)
Healthy controls	30	186.03±23.14
Breast cancer patients		
Stage II before chemotherapy	30	272.63±17.06[a]
Stage II after 1st cycle of chemotherapy	30	396.63±18.60[ab]
Stage III before chemotherapy	30	320.60±22.50[ab]
Stage III after 1st cycle chemotherapy	30	464.80±22.48[ac]

The data was expressed as Mean ± SD. [a] P<0.0001 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-4
Correlation analysis of measured parameters after 1st chemotherapy cycle

Parameters	After 1st chemotherapy cycle in stage II of disease.	After 1st chemotherapy cycle in stage III of disease.
GSH/GST	r= + 0.86	r= + 0.89
GSH/GPx	r= + 0.89	r= + 0.94
GSH/Cyt. C	r= - 0.89	r= - 0.98
GST/Cyt. C	r= - 0.97	r= - 0.92
GPx/ Cyt. C	r= - 0.98	r= - 0.91

This may cause apoptosis resistance in cancerous cells following chemotherapy[6]. 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 Czeczot H. et al[20] 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[10,21]. 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 1st 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[3,22]. 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[12].

Reduced glutathione besides its role as scavenger of free radicals also acts in association with the detoxification enzymes glutathione peroxidase (GPx) and glutathione-s-transferase (GST). These enzymes protects cells 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[13,23]. This antioxidant function of reduced glutathione along with its associated enzymes however, may provide protection against oxidative stress mediated apoptosis[6,24]. The levels of GST and GPx in serum were found to be elevated in most of the human cancers studied[15,23,24.] The post-operative higher values of GST were reported in patients with breast cancer and cancer of digestive tract[9, 25]. In this study, we found significantly higher values of serum GST and GPx 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[9,25]. The higher values were observed post-operatively might be because normalization of GST[25] and GPx levels after surgery may take about a month. Further, we found significant decrease in the serum levels of GST as well as GPx after 1st 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[12]. A significant positive correlation was observed between serum levels of GSH and GST as well as GSH and GPx after 1st cycle of chemotherapy. The decreased levels of both these enzymes observed in the present study may be related with decrease in GSH after chemotherapy. GST and GPx may utilize GSH in detoxification of highly reactive electrophiles produced during cytotoxic action of chemotherapy drugs[12].

The oxidative stress produced by antineoplastic drugs, as described by depletion of antioxidants, mainly activates the intrinsic pathway of apoptosis in cancerous cells, which induces opening of the mitochondrial permeability transition pore. This causes the translocation of cytochrome c from mitochondria to cytosol[26]. Berczyk et al[27] have shown that cytochrome c is released from mitochondria to cytosol during process of apoptosis and it further leaves the cells and can be detected in circulation. The elevated serum cytochrome c levels have been

reported previously in hematological malignancies, systemic inflammatory response syndrome and fulminant hepatitis[28-30]. In the present study, we found significantly higher serum cytochrome c levels 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. The higher value of cytochrome c in post-operative breast cancer patients before chemotherapy may indicate increased cell turnover and augmented spontaneous apoptosis[28]. Berczyk et al[27] and Renz et al[28] have reported that serum cytochrome c is an apoptotic marker in vivo and it reflects the cell death burden which is induced by cytotoxic drug therapy. Our observation of significantly elevated serum cytochrome c in stage II (P<0.0001) as well as stage III (P<0.0001) of the disease after 1st cycle of chemotherapy as compare to levels before chemotherapy correlates well with their reports [27, 28]. We found a significant inverse correlation between serum GSH and cytochrome c, serum GST and cytochrome c as well as serum GPx and cytochrome c after 1st cycle of chemotherapy. The observed increase in serum cytochrome c after 1st cycle of chemotherapy in the present study might be due to aggravated oxidative stress, as reflected by depleted antioxidants such as GSH, GST and GPx, that induces apoptosis in cancerous cells. The elevated serum cytochrome c level after chemotherapy is thus positive indicator of responsiveness of breast cancer patients to chemotherapy.

Conclusion

Our data suggests that administration of chemotherapeutic drugs causes depletion of antioxidants such as GSH, GST and GPx, in order to counterbalance the oxidative stress. Increased oxidative stress due to chemotherapeutic drugs causes apoptotic demise of cancerous cells which results in release of cytochrome c in the blood stream. Hence the increased level of apoptotic marker, cytochrome c is an indicator of therapy induced cell death burden. So the evaluation of its level may help clinicians to predict effectiveness of chemotherapy. However, this is a preliminary report. This work will be repeated with larger study group to confirm the results.

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