ABSTRACT

Background: Cisplatin and etoposide has a anticancer activity against various types of tumors. Cardiotoxicity by both drugs is dose dependent. The use of this drugs is often limited due to its side effects that includes silent and symptomatic arrhythmias. Studies are performed with an aim to study the effect of cisplatin and etoposide on heart. Methods: Rats were divided into three groups. Group 1 and 2 was injected with 0.4 mg of Cisplatin and, 1.0 mg of Etoposide per kg, i.p daily for 8 weeks. Control rats received 0.5 ml of saline daily. Glutathione (GSH), Glutathione-S-Transferase (GST), Glutathione Reductase (GR), Glutathione Peroxidase (Gpx), Gamma Glutamyl Transpeptidase (GGT), Catalase (CAT), Cytochrome p450 (Cyp450) and Lipid peroxidation Lpx (MDA content) was studied in all three groups. Protein studies were done by SDS-PAGE. Results: Reports obtained from our studies indicates that Cisplatin and Etoposide increases GSH levels, and GST, GR, Gpx, GGT, and Cyp450 activities. Both the drugs at its dose produced an increase in CAT activity and Lipid peroxidation Lpx (MDA Content) compared to controls. Sirtuin1(SIRT1), a NAD dependent deacetylase in cisplatin and etoposide treated groups exhibited an increase compared to controls. Conclusions: Cisplatin and Etoposide at low dose shows an increase in GSH and GSH-dependent enzymes that might protect heart from any oxidative damage. Decrease in CAT activity and Lpx (MDA content) indicates decrease in free radicals induced cardiotoxic effect. Thus it can be said that drug regimen might replenish NAD+ levels to normal which may protect the heart from any free radical induced oxidative damage.

KEYWORDS: Cisplatin; Etoposide; Lipid peroxidation; Cardiotoxicity.

INTRODUCTION

New strategies of anticancer drug therapies have shown cardiovascular side effects whereas, old anticancer therapies provide us with delayed serious complications in long term cancer survivors. An improvement in cancer treatment has led to new field the cardio-oncology. It is important to study the mechanism of action of drugs, mechanism of cardiotoxicity, and use of multi-drug resistance-targeted therapy.\cite{1,2}

Cisplatin is a potent anticancer drug that is associated with several side effects such as myelotoxicity, gastrointestinal toxicity, neurotoxicity, otoxicity, cardiotoxicity and nephrotoxicity. Cisplatin is a known nephrotoxicant however, cardiotoxicity by cisplatin at low dose has been least studied. We have previously documented the mechanism of action of cisplatin in hepatic and renal tissue. Both histopathological and antioxidant status have shown significant alterations.\cite{3,4}

Etoposide is a podophyllotoxin commonly been used for the treatment of refractory testicular tumors and small-cell lung carcinoma. The most common cardiac side effect of etoposide is hypotension, although myocardial ischemia and infarction have also been noted. The mechanism of action of the drug shows generation of semiquinone radicals, and phenoxyl radicals that have also been reported to have antioxidant action.\cite{5,6} The metabolic conversion of anticancer drugs, alkylating drugs, anthracyclines can generate reactive oxygen species (ROS) i.e., H2O2 from O2- , semiquinone or phenoxyl radicals at lower levels that can induce significant levels of antioxidant enzymes.\cite{7,8,9} ROS can also induce cell signalling pathways. Several reports have also stated that H2O2 have an ability to induce
HDAC proteins, SIRT1 and antioxidant program. Present studies are carried out with an aim to determine the role of cisplatin and etoposide on cardiac antioxidant enzymes followed by determining the LPx in male rat.

MATERIALS AND METHODS
Animals and Ethical Clearance
Adult male albino rats of Wistar strain were used for the study. Animals were weighing about 220-250g obtained from Rajudyog biotechnology division Maharashtra, India were used. The animal studies were carried out upon institutional animal ethical committee approval.

Drugs
Cisplatin, Etoposide was procured from Dabur India Ltd. All the chemicals, Bovine serum albumin (BSA), reduced and oxidized GSH, thio barbituric acid (TBA), butylated hydroxytolune (BHT), L-gammaglutamyl-p-nitro-anilidehydrochlorede, sodium azide, Dinitro thio benzoic acid (DNTB), 1-Chloro 2,4 Dinitro benzene (CDNB), NADPH, sodium dithionite, sodium formate were purchased from SD fine and SRL chemicals, India.

Methodology
Animals were acclimatized for a period of two weeks and were then treated. They received standard pellet and water ad libitum. Rats were coded in groups of two per cage and then were subsequently examined for further study. Experimental rats were injected with 0.4 mg of Cisplatin and, 1.0 mg of Etoposide per kg i.p daily for a period of 8 weeks. Control rats received 0.5 ml of saline daily along with the treated set of the rats. The change in the body weight was monitored per week.

Antioxidant and Lipid Peroxidation (MDA Content) Studies
At termination, rats were sacrificed using ether anaesthesia. Cardiac tissue was dissected out, washed in ice-cold saline, blotted and a homogenate was prepared in 0.1M sodium phosphate buffer (pH 8.0). Homogenate was further centrifuged and the supernatant fractions obtained were utilized for the analysis of GSH-related enzymes like GST, GR, Gpx, GGT, and CAT. Trichloroacetic acid (TCA) treated samples were utilized for the estimation of reduced GSH and LPx. The resulting supernatant fractions were recentrifuged for an additional 60 min at 105,000g. The microsomal pellets obtained were carefully collected and used for the estimation of CYP. Total protein content was estimated by Lowry et al. (1951). Sirt1 activity was determined by using N-acetyl Le-Lysine as substrate was estimated by Smith (2009). All spectrophotometric readings were taken on Shimadzu UV-160 double beam spectrophotometer.

Analysis
The significance of difference between the means was calculated by students t-test and ANOVA for three groups are used as appropriate. Results were expressed as mean ± SEM. Significance between groups controls, cisplatin and etoposide is shown as *p<0.05

Protein Extraction & Separation on Sodium dodecyl sulphate and polyacrylamide (SDS-PAGE) Gel Electrophoresis
SDS-PAGE studies for control, cisplatin and etoposide heart was carried out for separation of proteins. The studies would elucidate any drug effect on the heart. Cardiac tissue was dissected out, washed in ice-cold saline, blotted and a homogenate was prepared in lysis buffer (50mM Tris-cl,pH 7.5, 150mM Nacl,2mM EDTA, 0.5mM DTT, 1.0mM PMSF) followed by addition of 1% Triton X-100 and 2% NP-40 with final concentration of 5ml/gm tissue with lysis buffer. Samples were sonicated for 6 cycles of 30secs pulse with intermittent cooling for 60secs on ice. After sonication samples were centrifuged at 10,000 rpm for 30mins, at 4°C. Cell fractions used for determining protein concentration by Folin Lowry (1951). Standard molecular weight protein marker's ranging from 66kd-14.2kd were used.

Preparation of SDS-PAGE Gel and Sample Preparation
For SDS-PAGE gel electrophoresis studies 5% Stacking gel and 12% Resolving gel was prepared and used for separation of proteins. Experimental groups containing Cisplatin, Etoposide and Control heart samples having 20-50 µg of protein were used for loading. All three samples were treated with equal volume of tracking dye (0.05M Tris-cl, pH 6.8, 2% SDS, 10% glycerol, 0.25% bromophenol blue as a indicator dye).

SDS reducing buffer was prepared by adding 50µl of β-mercaptoethanol to 1ml of stock solution as 2x tracking dye. Samples were treated for 5 mins, 95°C on heating block followed by quenching on ice and centrifuged at 10,000 rpm, 5 mins. Standard molecular weight protein marker used were obtained from sigma chemicals, USA. The molecular weight of these proteins are as follows: BSA (66kd), ovalbumin (45kd), glyceraldehyde-3-phosphate dehydrogenase (36kd), carbonic anhydrase (29kd), trypsinogen (PMSF treated) (24kd), trypsin inhibitor (20kd), alpha-lactalbumin (14.2kd). All the samples along with standard molecular weight protein marker were electrophorized. A constant current of 60 mA was used till samples were stacked in stacking gel followed by 31mA till bromophenol blue marker reached at the bottom of the gel. The gel was removed by dismanteling unit and was subjected for silver staining.

Staining and Destaining of Gel
Fixation of gel was done by using Destaining I solution (50% methanol, 37µl formaldehyde, 12% Glacial acetic acid) for 20 mins. The gel was washed twice each in 50% ethanol. Inpregnate the gel with 1X Na2S2O3 solution for 1min. Gels were washed thrice in water and was soaked in silver nitrate (200mg/100ml distilled water, 37µl formaldehyde) for 30 min’s, which was then...
washed in distilled water for 15-20 seconds. The protein bands were developed using developer (6% Na₂CO₃, 2% Na₂S₂O₅, formaldehyde), for 2-5 min. The reaction was stopped by using 1% glacial acetic acid.

RESULTS

Body Weight
Total bodyweight of 8 weeks control, cisplatin and etoposide treated animals are shown in Fig 1A. Cisplatin and Etoposide treated rat registered significant decrease in body weight shown as, 188±0.1 and 304±14.352 when compared to controls, 367±12.31.

Indicator of Oxidative Stress Lipid Peroxidation (MDA content) and Cholesterol levels of heart
Fig 1B & C depicts total protein content and Lpx (MDA content) values of heart. The mean values were expressed as mg/gm tissue. Total protein content registered significant increase for cisplatin treated heart shown as, 321±12.6 and etoposide as 423±15.8, as compared to controls reported as 259±14.1 (fig. 1B). Lpx (MDA content) was decreased compared to control rats. Cisplatin and etoposide treatment showed significant decrease reported as 0.3±0.01 and 0.4±0.03 as compared to controls 0.61±0.06 (fig. 1C). Cholesterol levels in heart showed significant increase in cisplatin rats (2.452±0.05) and decrease in etoposide rats (1.44±0.07) as compared to controls 0.758±0.03 (fig. 1D).

Antioxidant Studies
The mean values of GSH in cisplatin and etoposide treated heart showed significant increase, 9.567±0.06, 8.59±0.03 compared to control 6.53±0.06 (fig. 2A). Cisplatin and etoposide treatment to rats showed significant increase in GST activity in heart, 5.79±0.05 and 3.432±0.47 as compared to controls reported as, 1.25±7.4 (fig. 2B). The mean values of GR activity in cisplatin and etoposide heart tissue reported significant increase shown as 7.28±0.06 and 8.37±0.07 as compared to control counterpart 5.63±0.25 (fig. 2C). GPx activity showed as 198±0.79, 175±0.06 in cisplatin and etoposide treated heart compared to control heart shown as 165±0.5 (fig. 2D). A significant increase in GGT activity in cisplatin and etoposide groups, 9.5±0.07 and 9.94±0.06 as compared to the controls group 8.096±0.06 (fig. 2E).

The level of drug metabolizing enzyme namely CYP450 in heart showed significant increase as 689±70 and 621±82 in cisplatin and etoposide treated rats as compared to control rats 587±0.406 (fig. 2G). SIRT1 activity, is NADPH dependent deacetylase shown an increase in activity in cisplatin and etoposide treated heart, registered as 25.67±0.04 and 14.45±0.006 as compared to control 2.56±0.005 (fig. 2H).

SDS-PAGE Protein studies
SDS-PAGE gel studies shows the protein patterns with molecular weight of 66 kd were least expressed in etoposide treated heart (Lane 3, 7, 10, 11), as compared to marker (Lane 6) and controls (Lane 2, 4, 8). Fig.3 shows overexpression of control (Lane 2, 4, 8) and cisplatin treated heart protein (Lane 1, 5, 9, 12) with a range from 66-14.2kd. Etoposide treated heart showed downregulation of protein pattern ranging from 45, 36 kd compared to marker and control rat (fig. 3). Marker (Lane 6) (66kd, 45kd, 36kd, 29kd, 24kd, 20kd, 14.2kd)
Figure Legends

Fig. 1: General Health and Indicator of Oxidative Stress Lipid Peroxidation Lpx (MDA Content)
The experimental set up included treatment of male rats with Cisplatin (0.4 mg/kg body wt, daily,i.p) and Etoposide (1 mg/kg body wt, daily,i.p) for 8 weeks. Animals did not show any mortality rate during the entire period of treatment. Body weight was significantly decreased in cisplatin and etoposide treated rats compared to control counterpart (1A). Total protein...
content in cisplatin and etoposide treated group was increased as compared to control rats (1B). Lipid peroxidation LPx (MDA content) were significantly reduced in cardiac tissue in cisplatin and etoposide groups (1C respectively) and Cholesterol levels was increased in both the drug treated rats as compared to control counterpart (1D respectively). Values are expressed as mean ± SEM (n=5). Statistically significant changes between untreated and treated sets are marked as *(P<0.05).

Fig. 2: Regulation of GSH, GST, GR, Gpx, GGT, CAT, CYP450 antioxidant enzymes and SIRT1 activity
Rats treated with Cisplatin (0.4 mg/kg body wt, daily,i.p) and Etoposide (1 mg/kg body wt, daily,i.p) for 8weeks showed significant increase in cardiac antioxidants GSH and GSH-dependent enzymes GST, GR, Gpx, GGT activity as compared to controls (2 A,B,C, D & E respectively). CAT activity showed significant decrease in cisplatin and etoposide treated rats compared to control counterpart (2F). The drug metabolizing enzyme CYP450 activity was increased in cisplatin and etoposide heart tissue compared to controls (2G). Increase in SIRT1 activity in cisplatin and etoposide treated heart compared to controls (2H).

All the values are expressed as mean ± SEM (n=5). Statistical significant differences between untreated and treated rats are marked as *(P<0.05).

Fig. 3: Protein Expression in Cisplatin and Etoposide treated rats
Rats treated with Cisplatin (0.4 mg/kg body wt, daily,i.p) and Etoposide (1 mg/kg body wt, daily,i.p) for 8weeks showed significant alterations in different molecular weight protein expression of heart compared to control counterpart. Fig 3 shows silver staining of SDS-PAGE of Cisplatin and Etoposide treated heart protein extract. Cisplatin heart extract (Lane 1, 5, 9, 12), Control (Lane 2, 4, 8) and Etoposide heart extract (Lane 3, 7, 10, 11). Marker (Lane 6) (66kd, 45kd, 36kd, 29kd, 24kd, 20kd, 14.2kd).

DISCUSSION
Numerous anticancer drugs are known to induce cytotoxic action towards neoplastic cells often leads to apoptosis and phagocytosis without induction of any inflammation.[21,22] The mechanism of action of cancer drugs generates free radicals by oxidation-reduction reaction which are also responsible for depletion of antioxidants. Kharbhagare et al., (2000) reported that cisplatin treatment schedule is associated with increased risk of causing long-term toxicities that includes nephrotoxicity, otoxicity and neurotoxicity. Cisplatin can also cause depletion in ATP levels which can affect significant decrease in synthesis of glutathione.

Chan at.el., (2002) reported that mitochondria contributes to the regulation of energy production, metabolism, redox status and apoptosis. An enhanced mitochondrial membrane potential (MMP) is prevalent to cancer cell phenotype and lipophilic cations accumulation inside mitochondria is a consequence of the higher membrane potentials. We have carried out an analysis on antioxidant status and biomarkers along with lipid peroxidation in cardiac tissue of rat after cisplatin and etoposide treatment. Cisplatin and Etoposide therapeutic regimen at given dose produced significant increase in GSH levels followed by increase in GST, GR, Gpx and GGT activity in cardiac tissue of rat. The studies indicates that both the drugs, might have not produced any free radical induced oxidative stress to heart which is evidant by significant decrease in LPx (MDA content) as compared to control rats. The decrease in catalase activity further suggest that the mechanism of action of cisplatin to aquated/hydroxylated form by cytochromeP450 system might have generated low levels of ROS (Scheme 1).

Our Scheme explained the mechanism of action of Cisplatin to aquated-Cisplatin species via; cytochromeP450. The activated aquated-Cisplatin species may act as a inducer of GSH-related enzymes GST, GR, Gpx and GGT increasing the GSH status in heart. Cisplatin and GSH can further metabolize to form GSH-Cisplatin conjugate, Cisplatin-S*, and Cisplatin-OS* species that might also enhance enzymes activity. The reactions also induced Sirt1 that might deacetylante antioxidant redox enzymes (ARE) NRF1 and NRF2 increasing the thiol status in heart. An increase in thiols can increase cell resistance/cellular efflux of cisplatin which might lower cardiotoxicity, decrease oxidative stress (OS), and diminish DNA platination. We also report here the formation of superoxide anion radical (O) and by dismutation forming hydrogen peroxide (H₂O₂) at lower level which can act as a inducer of antioxidant enzymes and Sirt1 (Scheme 1).

Similarly, etoposide mechanism of action by cytochromeP450 system generated lower levels of phenoxyl or semiquinone radicals that can serve as inducer of GSH and GSH related enzymes. The depleted catalase activity further supports the findings. There are studies done on etoposide reported that phenoxyl radicals can also produce antioxidant action.[23] Thus it can be said that low levels of ROS can serve “purposeful” roles as “regulators” of cell function or as “signalling molecules”. [25]

Toxicity of a compound may derive from its metabolism and unwanted ‘‘random’’ protein interactions. Abundant cysteine-rich small peptides, especially glutathione (GSH), and proteins such as metallothionein represent detoxifying that would deactivate pathways for inorganic drugs. [26]

Our studies shows that cholesterol levels were significantly increased in both groups but did not seen to produce any crucial effect to heart. It has been reported
that cisplatin resistance is multifactorial and is combined with both decreased cellular accumulation of cisplatin, increased efflux of platinum from the cell and increased cytoplasmic detoxification (through increased levels of cellular thiols such as GSH). The fate of metabolism of cisplatin is the formation of mono- and bisqua species cis-[Pt(NH and cis[Pt(NH3)2(H2O)2]+ that can act as a inducer of antioxidants enzymes. Thus, we assume that an increased GSH status in cardiac tissue is a reason of decreased toxicity and a rise in thiol may also increase the drug efflux. In one studies it has been reported that GSH competes for platinum binding and diminishes the DNA platination, thus reducing cytotoxicity. Thannickal VJ, et. al. 2000 reported that exogeneous H2O2 (usually in the millimolar range) has been shown to induce tyrosine phosphorylation and activation of the PDGF-α, PDGF-β, EGF receptors. Furthermore, lower levels of H2O2 generated might also be responsible for inducing SIRT1+, thus increase in activity could be a significant effect of cisplatin and etoposide drug in heart. There are several reports which suggest that H2O2 can act as an activator of HDACetylases that includes SIRT 1 in cardiac myocytes. The increase in Sirtl activity can participate in increase in antioxidant status GSH levels. An increase in ROS can play an important role in energetic recovery which might be required during myocardial ischemia, myocardial infarction and cardiomyopathy. Thus cisplatin and etoposide at given dose might increase the GSH synthesis by inducing GST, GR, Gpx and GGT that might further enhance the adenosine-triphosphate (ATPase) activity, and increase ATP levels in heart.

Recent studies of cardioprotective effect of tadalaafil (Tad) on cisplatin has been explained by Saleh RM et.al (2015) in his studies. There data suggests that Tad reduced blood pressure, heart rate, levels of serum cardiac troponin (cTn-I), and malondialdehyde (MDA), and combination treatment of Tad and cisplatin increased levels of reduced glutathione (GSH) and nitric oxide (NO) in the heart treated rats. Furthermore, Gi-Su Oh et.al (2016) have reported decrease in intracellular NAD+/NADH ratio is cause of cisplatin-induced kidney damage through inflammation and oxidative stress however, an increase in cellular NAD+/NADH can suppress the damage. Thus NAD+ dependent cellular pathways can produce beneficial action. Thus, we assume in our studies that cisplatin and etoposide treatment as monotherapy at given dose might be replenishing NAD+ levels in heart protecting organ from any damage which is evident by decreased MDA levels. Studies by Jamshidzadeh A et. al (2016) reported that eisenia foetida a glycoprotein extract can prevent cisplatin induced nephrotoxicity.

Studies on SDS-PAGE depicts, protein expression in cisplatin, control and etoposide treated heart. Figure 3 reveals protein patterns with molecular weight of 66 kd were underexpressed in etoposide treated heart as compared to marker and controls. In control and cisplatin treated heart protein range from 66-14.2kd seems to be overexpressed, however, in etoposide treated heart showed least expression of proteins that ranges from 45, 36 kd compared to marker and control rat.

The molecular weights of GST (mol.wt- 45,000), GR (mol.wt- 97 kd), Gpx-1 monomer (mol.wt- 23 kDa), Gpx-1 homotetramer: (mol.wt-92 kDa), Gpx-3: (mol.wt-kDa), Gpx-5: (mol.wt-26 kDa).

Therefore from present studies it can be predicted that in control and cisplatin heart tissue GST might be overexpressed, but in etoposide group, GST shows lower expression. Gpx expression is higher in control and cisplatin rats, but lower in etoposide rats. Literature on Gpx reveals that under regulation by hypoxic stress and the expression and deficiency of Gpx-3 is associated with cardiovascular disease and stroke.

Additionally, GGT molecular weight of GGT1 isoforms (mol.wt-61/39/24 kDa), GGT2 isoforms: (mol.wt- 62/61 kDa). In all three groups, Cisplatin, control and etoposide the previous two groups shows expression (i.e. 39 and 24kd), but etoposide shows least expression compared to control, cisplatin and marker. Catalase (mol.wt-60 kDa) in heart of control, cisplatin and etoposide rats did not show any expression, which is also in support to the biochemical studies of catalase activity. Thus, altogether it can be said that all the proteins whether increased at biochemical level or overexpressed or expressed at lower level seen by SDS-PAGE can induce antioxidant effect and can prove to be beneficial in heart function.

CONCLUSION

Cisplatin and Etoposide mechanism of action can increase various antioxidant program that can enhance cardiac cell resistance against drugs, lower cell toxicity and prevent cell platination in cisplatin therapy. In addition cisplatin and etoposide conjugates and further formation of hydrogen peroxide at lower levels can acts as a inducers of Antioxidant reduct (AOX) and HDAC protein. Thus both the drugs given as a monotherapy agent may prove beneficial action to cardiac tissue while given with any antioxidant might nullify the drug action.

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