Protective effect of *Gingko biloba* extract against doxorubicin-induced cardiotoxicity in mice

M U R Naidu, K Vijay Kumar, I Krishna Mohan, C Sundaram & Shashi Singh

Central Research Laboratory, Departments of Clinical Pharmacology & Therapeutics, & Pathology, Nizam’s Institute of Medical Sciences, Hyderabad, Centre for Cellular and Molecular Biology, Hyderabad 500 082, India

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Doxorubicin (DXR) causes dose dependent cardiotoxicity in experimental animals and in humans. In chronic doxorubicin cardiotoxicity model mice, the role of *G. biloba* extract (Gbe) which has an antioxidant property, was investigated. Doxorubicin treated animals showed higher mortality (68%), increased ascites, marked bradycardia, prolongation of ST and QT intervals and widening of QRS complex. Myocardial SOD and glutathione peroxidase activity were decreased and lipid peroxidation was increased. Ultrastructure of heart of DXR treated animals showed loss of myofibrils, swelling of mitochondria, vacuolization of mitochondria. *G. biloba* extract significantly protected the mice from cardiotoxic effects of doxorubicin as evidenced by lowered mortality, ascites, myocardial lipid peroxidation, normalization of antioxidant enzymes, reversal of ECG changes and minimal ultrastructural damage of the heart. The results indicate that administration of *G. biloba* extract protected mice from doxorubicin-induced cardiotoxicity.

Anticancer therapy is not only cytotoxic to cancer cells, but also to healthy cells. This results in a narrow therapeutic index; with adverse events frequently limiting the administration of optimal anticancer therapy. The anthracycline antibiotics, including doxorubicin (adriamycin), daunorubicin and epirubicin are among the most active anticancer agents used against a wide range of solid and haemopoietic malignancies. Anthracycline-induced cardiotoxicity limits effective clinical use of these compounds. Cardiotoxicity may occur acutely with electrocardiogram changes, arrhythmias, or a myocarditis-pericarditis syndrome. The most serious form is a chronic cumulative dose-related cardiomyopathy.

The mechanism by which anthracines cause cardiotoxicity and antitumor activity are thought to be different. Cardiotoxicity is thought to result from several mechanisms. The anthracycline cardiotoxicity appears to be associated with the generation of reactive oxygen species. Anthracyclines contain a quinone group, which undergoes electron reduction to produce a semi-quinone radical. Interaction of oxygen with this semi-quinone initiates a cascade of reactive oxygen species. In addition, the anthracycline-iron complex is a powerful oxidant itself and is able to initiate lipid peroxidation in the absence of oxygen free radicals. Anthracycline-induced injury may be multifactorial and complex. One mechanism common to most of these is the increased oxidative stress.

Initial attempts at preventing anthracycline-induced cardiac damage focused on the oxygen free radical scavengers. Studies with acetylcysteine and alpha-tocopherol did not demonstrate any cardiac protection in humans. Amifostine by virtue of its potential to scavenge oxygen radicals, has been shown to reduce doxorubicin-associated cardiotoxicity in cultured neonatal myocytes and in mice. Monohydroxy ethylrutoside, a semisynthetic flavonoid has been shown to reduce doxorubicin cardiotoxicity in vitro and in mice. *In vitro* studies have demonstrated that *G. biloba* extract (GBe) scavenges free radicals. GBe protected the human liver microsomes from lipid peroxidation caused by cyclosporine. The extract of *Gingko biloba* leaves (Egb-761) is a mixture of flavonoid glycosides and other natural compounds. Flavonol glycosides and proanthocyanidine present in GBe have significant oxygen free radical scavenging activity. In our recent study, GBe through its antioxidant property protected the rats from gentamicin-induced nephrotoxicity. The aim of the present study was to investigate the effect of GBe against doxorubicin-induced cardiotoxicity in mice.

**Materials and Methods**

**Animals and treatment**—Female Swiss albino mice (mean weight 30-40 g) were housed under conditions of controlled temperature and 12 hr lighting cycle and fed standard diet. The animals were divided
into four groups of 16 animals each. The control animals received normal saline intraperitoneally once weekly for 4 weeks. The second group received four equal injections (each containing 4 mg/kg BW doxorubicin) intraperitoneally, once weekly for four weeks (cumulative dose 16 mg/kg) and the third group received GBe (100 mg/kg) orally, every day for 4 weeks along with four injections of doxorubicin similar to second group. All animals were observed for 3 weeks after the last injection of doxorubicin pertaining to their body weight, general appearance, electrocardiograph (ECG) changes and mortality. At the end of 3rd week the animals were sacrificed and the heart tissue was evaluated for lipid peroxidation products, antioxidant enzymes and morphological appearance. This study protocol was approved by the Ethics Committee on animal experimentation of the Nizam’s Institute of Medical Sciences, Hyderabad.

**Electrocardiograph**

Before sacrificing the animals, ECG was recorded under mild ether anesthesia. For ECG recording, needle electrodes were inserted under the skin for the limb leads at position II and a Grass polygraph, USA was used. The ECG tracings were recorded at the paper speed at 100 mm/sec. Heart rate, ST interval and QT interval were determined from the ECG.

**Lipid peroxidation products**

The degree of lipid peroxidation in heart tissue was estimated by measuring TBARS as described previously. Portions of heart tissue were dissected and homogenized in PBS, pH 7.4. The homogenate were centrifuged and the supernatants were collected and precipitated with 20% trichloroacetic acid and centrifuged. To the protein free supernatant, 0.33% thiobarbituric acid was added and boiled for 1 hr at 95°C; the TBA reactive products were extracted in butanol and the intensity of the pink color was read at 520 nm. Freshly diluted tetramethoxy propane (Sigma Chemical Co., USA) which yields malondialdehyde (MDA) was used as standard and data was expressed in nmol of MDA/g of heart tissue.

**Estimation of antioxidant enzymes**

Superoxide dismutase (SOD) was estimated in the heart tissue homogenate by cytochrome C reduction in a xanthine-xanthine oxidase generating system. SOD activity was determined from a standard curve of percentage of inhibition of cytochrome C reduction with a known SOD activity. Catalase activity was determined by the method as described earlier, with \( \text{H}_2\text{O}_2 \) (10 mM) and phosphate buffer (0.05 M, pH 7.0) at 210 nm. A unit is defined as the amount of enzyme that catalyzed the dismutation of 1 μmole of \( \text{H}_2\text{O}_2 \)/min. The specific activity is expressed in units/mg protein. Glutathione peroxidase activity was measured by the NADPH oxidation method and expressed as nmol of NADPH oxidized to NADP/mg protein. Protein was determined by the method as described earlier.

**Total antioxidant activity**

The total antioxidant activity in plasma and heart tissue was measured by decolorization assay. The pre-formed radical monocation of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS\(^{+}\)) is generated by oxidation of ABTS (Sigma Chemical Co., USA) with potassium persulfate and is reduced in the presence of hydrogen-donating antioxidants. Trolox (6-hydroxy-2,5,7,8-tetramethylicroman-2-carboxylic acid; Aldrich Chemical Co., Gillingham, Dorset, UK), a water soluble vitamin-E analogue was used as a standard.

**Electron microscopic studies**

For ultrastructural studies, 3 to 5 hearts in each group were processed. Fixation of samples in 3% glutaraldehyde in 0.1 M phosphate buffer for 3 hr at room temperature. Washed with PBS four times. Post fix in 1% \( \text{CrO}_4 \) in 0.1 M Phosphate buffer for 3 hr followed by washing in 0.1 M Phosphate buffer. Dehydrate in ascending grades of acetone, infiltrate in propylene oxide:resin 1:1 mixture overnight. Embedded in pure resin and use at 60°C for 72 hours. 30-50 nm sections were picked up on Cu grids, stained in uranyl acetate and lead citrate and scanned in JEOL 1000 CX electron microscope.

**Statistical analysis**

Results are presented as mean ± SD. The significance difference among the four groups was assessed using one way ANOVA and TUKEY’S HSD test to identify differences between the groups. Statistical significance was acceptable to a level of \( P<0.05 \).

**Results**

Intraperitoneal administration of DXR at the total cumulative dose of 16 mg/kg (4.0 mg/kg once per week for 4 weeks) induced clear signs of cardiac toxicity in mice. Doxorubicin treated animals appeared
more sick, weaker and lethargic compared to control and GBe+DXR group. Animals belonging to DXR group developed distention of the abdomen due to marked ascites, and when sacrificed, had a significantly more amount of peritoneal fluid (1.95±0.7 ml) compared to the DXR + GBe (0.52±0.3 ml) (Table 1). In addition to ascites the liver was enlarged and congested in all the mice who received DXR. During the 3 weeks posttreatment period, the mortality was significantly higher in the DXR group (68%) compared to GBe plus DXR group (36%) (Table 1). No deaths were observed in control or GBe group. Treatment with DXR resulted in a significant decrease in heart weight (47%) and heart to body weight ratio (37%) compared to control. In GBe + DXR group, heart to body weight ratio was similar to that of control and GBe treated group.

ECG changes
Intraperitoneal administration of DXR produced ECG changes characterised by bradycardia, prolongation of ST and QT intervals (Table 2 and Fig. 1). The mean heart rate in DXR-treated mice reduced to 552 ± 66 bpm than control (760 ± 82 bpm) The ST and QT intervals increased to 46 ± 26 ms and 56 ± 1.5 ms with doxorubicin respectively. GBe treatment antagonised DXR-induced bradycardia and prolongation of ST and QT intervals.

Antioxidant enzymes and TBARS products
The product of lipid peroxidation measured as TBARS in heart tissue was significantly higher in DXR treated group (30 ± 2.5 nmoles/g of heart tissue) compared to control (19.8 ± 0.6 nmoles) (Table 3). Co-administration of GBe with DXR produced a significant decrease in TBARS (22 ± 1.0 nmoles/g of heart tissue). There was no change in plasma TBARS in all the treated group indicating that DXR induced oxidative stress was limited to heart tissue. G. biloba extract, GBe plus DXR treated group showed significant increase in total antioxidant activity (Table 3). Different antioxidant enzymes were examined in heart tissue in all the groups and data are shown in Table 3. DXR produced significant decrease in SOD (24 ± 3.6 U/mg protein), glutathione peroxidase (34 ± 7.2 nmoles/mg protein) compared to controls. In controls, the activities of SOD and glutathione peroxidase were 37 ± 7.5 U/mg protein and 55 ± 9 nmoles/mg protein respectively. The activities of SOD and glutathione peroxidase in GBe+DXR group were comparable to control.

Electron microscopic studies
Morphological appearance of mitochondria, sarcoplasmic reticulum, sarcomeres, myofibrils and intercalated disks from control and GBe treated mice were normal. Hearts from DXR treated animals show loss of myofibrils, swelling of mitochondria, vacuolization of the cytoplasm and dilation of the sarcotubular system. In addition to swelling of mitochondria, there was disarrangement and disruption of cristae (Fig. 2b). The ultrastructure of hearts from GBe+DXR group showed minimal dilation of the

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Doxorubicin</th>
<th>GBe</th>
<th>DXR + GBe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mortality (%)</td>
<td>Nil</td>
<td>68%</td>
<td>Nil</td>
<td>36%</td>
</tr>
<tr>
<td>Ascitic fluid volume (ml)</td>
<td>Nil</td>
<td>1.95 ± 0.78*</td>
<td>Nil</td>
<td>0.52 ± 0.3**</td>
</tr>
<tr>
<td>Heart weight (mg)</td>
<td>197.00 ± 14</td>
<td>105.00 ± 18*</td>
<td>195.00 ± 8.40</td>
<td>150.00 ± 22**</td>
</tr>
<tr>
<td>Heart/body wt ratio x 10²</td>
<td>5.40 ± 0.1</td>
<td>4.20 ± 0.4*</td>
<td>5.40 ± 0.21</td>
<td>5.30 ± 1.0**</td>
</tr>
</tbody>
</table>

*P < 0.05 vs control group; **P < 0.05 vs doxorubicin group.

Table 2 — Effect of G. biloba extract on doxorubicin-induced changes in ECG in mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Doxorubicin</th>
<th>GBe</th>
<th>DXR + GBe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (bpm)</td>
<td>760.0 ± 82</td>
<td>552.0 ± 66*</td>
<td>756.0 ± 82</td>
<td>736.0 ± 76**</td>
</tr>
<tr>
<td>ST interval (msec)</td>
<td>13.2 ± 0.5</td>
<td>46.0 ± 2.6*</td>
<td>14.0 ± 0.6</td>
<td>18.6 ± 1.0**</td>
</tr>
<tr>
<td>QT interval (msec)</td>
<td>23.6 ± 0.8</td>
<td>56.0 ± 1.5*</td>
<td>24.1 ± 0.8</td>
<td>26.0 ± 9.0**</td>
</tr>
</tbody>
</table>

*P < 0.05 vs control; **P < 0.05 vs doxorubicin

DXR, doxorubicin; GBe, Gingko biloba extract.
sarcotubular system and occasional vacuolization of the cytoplasm (Fig. 2c).

**Discussion**

Cancer chemotherapy with anthracyclines of which DXR is the main representative is limited by its cardiomyopathy, developing in animals and patients after cumulative dosing. Intraperitoneal administration of DXR produced cardiotoxicity in mice, which is characterised by increased mortality, decreased body weight: heart weight ratio, and accumulation of ascitic fluid. In rats, DXR produced increased mortality and ascites as compared to control. Administration of GB lowered DXR induced mortality in mice. Similarly, lipid lowering drug and antioxidant, probucol and potent antioxidant melatonin has been shown to reduce doxorubicin-induced deaths in rats. Several authors have described the changes in ECG of laboratory animals with anthracylne and have demonstrated that, the severity of ECG changes paralleled with known doxorubicin-cardiotoxicity in man. In our experiment, DXR produced marked bradycardia, prolongation of ST and QT interval. Doxorubicin has profound influence on the shape of the ECG. The QT and ST intervals were increased, this is in accordance with other reports in animals. ECG changes noted in our experiment were similar to those reported in mice. GB was able to prevent the development of ECG changes induced by doxorubicin. Parachini and coworkers have demonstrated the protective effect of several spin traps by measuring changes in ST and QT interval in rats. Changes in ST interval have been found to correlate with degree of cardiotoxicity in rats and mice. In a study, Venoruton, which contains 10% of monomer, was able to completely prevent the cardiotoxicity in mouse. Both ICRF-187 a iron chelating agent and monomer antioxidant was able to protect against DXR-induced ECG changes.

Myocardial damage is specific to all anthracycline antibiotics, including myofibrillar degeneration, mitochondrial dilatation, cellular vacuolization and finally myocyte dropout. As seen in the present study, DXR treatment caused significant ultrastructural changes including marked myofibrillar loss, vacuolization and mitochondrial damage. In mice treated with GB, these DXR-induced ultrastructure changes were minimal suggesting protection of cellular damage by GB. In rats DXR exhibited myocardial lesions mainly cytoplasmic vacuolization, myofibrillar loss and swelling of mitochondria, disarrangement and mitochondrial degeneration, formation of lysosomal bodies, dilation of sarcotubular system. Doxorubicin-induced ultrastructural changes in rats treated with melatonin and probucol were almost indistinguishable from the control, with regular myofibril arrangement and weakly preserved mitochondria, only in few specimens, perimitocondrial edema was observed. Reduction in scores of myocardial lesions-induced by DXR was reported with Venoruton and ICRF-187. In mice, we also found similar in scores of myocardial lesion with GB.

In mice and rats, DXR significantly increased MDA levels in myocardial tissue. In our study,
Fig. 2—Ultrastructure of heart from (a) control mice treated only with normal saline; (b) myocardial tissue treated with doxorubicin showing loss of myofibrils (MF), swelling of mitochondria (M), vacuolization of cytoplasm (V) and dilation of sarco tubular system (S); (c) heart tissue treated with doxorubicin + G. biloba extract showing minimal dilation of the sarco tubular system and occasional vacuolization of the cytoplasm.
MDA levels with decrease in levels of DXR treated mice showed increase in heart tissue changes in MDA and enzyme levels. Increase in total antioxidant activity in plasma and heart tissue in GBe and GBe plus DXR treated groups may be due to the prevented by melatonin, a potent hydroxyl radical scavenger\(^2\). GBe scavenges free radicals\(^1\). Oxyl radical and as potent inhibitors of lipid peroxidation in rat microsomes and human liver microsomes. Flavonoids have long been recognized as scavengers of superoxide, hydroxyl and peroxyl radical and as potent inhibitors of lipid peroxidation\(^3-5\). The Folium Gingko contain a wide variety of phytochemicals, the major constituents are flavonoids that are based on flavonols, kaempferol and quercetin\(^6\).

In the present study the cardioprotective effect of GBe may be due to its flavonoid content. Monohydroxy ethylresorafen a semisynthetic flavonoid protect against DXR-induced cardiotoxicity, without influencing the antitumor activity of that drug in vitro and in vivo\(^7\). Similarly, amifostine by virtue of its potential to scavenge oxygen radical has been shown to reduce DXR-toxicity in rat heart myocytes\(^8\) and mice\(^9\). The protection from DXR-induced cardiotoxicity by GBe may also have some other mechanisms. The ginkgolides B is known antagonists of platelet activating factor (PAF)\(^1\). PAF is a potent inducer of oxygen free radicals and ginkgolides B specifically inhibited PAF-induced degranulation, superoxide generation and chemotaxis of neutrophils\(^1\). In isolated rat hearts, calciuminhibitor FK 506, the PAF antagonist plus G. biloba extract (EGb 761) synergistically produce cardioprotective effects\(^10\).

In conclusion, we observed good cardioprotective effect of GBe against chronic doxorubicin-induced cardiotoxicity in mice thus GBe merits further investigation as a possible cardioprotective agent against doxorubicin-induced chronic cardiotoxicity in patients.

Acknowledgement

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