Prophylactic Efficacy of a Combination of Proanthocyanidin and Vitamin E on Hepatotoxicity Induced by Doxorubicin in Rats

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Abstract

Doxorubicin (Dox) is a common chemotherapeutic anticancer drug. One of its side effect, according to some studies is oxidative stress-induced toxic changes on the liver. In this study protective efficacy of Proanthocyanidin (GSPE) along with vitamin E on Dox induced hepatotoxicity was evaluated in 50 male (divided in to five groups) Wister rats. After 2 weeks of the experiment and drug administration, blood samples and liver tissues were taken from all groups. Lipid profiles, liver function test in blood serum and Superoxide Dismutase (SOD), Catalase (CAT) and Malondialdehyde (MDA) levels in liver tissue were measured. We found that intraperitoneal administration of Dox had profound effects on the liver as assessed by liver function tests and liver histology. The present results showed that pretreatment with proanthocyanidin extract, along with vitamin E enhances the antioxidant defense against Reactive Oxygen Species and reduced the oxidative stress and change in activity of antioxidant enzymes induced by Dox, thereby demonstrating the antioxidant potential of GSPE along with vitamin E in ameliorating the hepatotoxicity induced by Dox and it shows that it could be one approach to reduce the risk of Dox induced side effects in clinical settings.

Keywords: Dox, Oxidative Stress, Hepatotoxicity, Reactive Oxidation Species, Antioxidant Enzymes, Lipid Peroxidation, Proanthocyanidin, Vitamin E

1. Introduction

The use of chemotherapeutic agents in cancer treatment is often accompanied by side effects due to oxidative stress. Increase lipid peroxidation, reduced antioxidant vitamins, free radical trapping capacity in plasma and a marked reduction of tissue Glutathione (GSH) levels are frequently reported during chemotherapy (Goncalves et al, 2009). The enhanced production of Reactive Oxygen Species (ROS) damages normal tissues and therefore results in toxic side effects of chemotherapeutic agents. In particular, tissue and cells with a high proliferation rate are most affected by the oxidative stress (Conklin, 2000). ROS generated during cancer chemotherapy may also decrease the efficacy of the treatment by interfering with drug induced apoptosis and cell cycle progression, which are optimal effect on cancer cells (Conklin, 2004 and Wessner et al, 2007). Doxorubicin (Dox) is one of the most widely used and successful chemotherapeutic anti-tumour drugs prescribed in haematological malignancies and solid tumours (Li et al, 2009). Its clinical application is limited due to its cumulative dose-related cell toxicity. Proposed mechanisms include the generation of reactive oxygen species-mediated oxidative stress. Therefore, reducing oxidative stress should be protective against Dox-induced cell death (Li et al, 2010). Injury and dysfunction induced by drug toxicity is an important marker and potentially contribute to the initiate-
on and progression of most forms of liver disease, leading to the development of hepatic lesions. Such repeated acute insults by cytotoxic drugs may contribute to a disease risk in healthy individuals and promote disease progression in established patients (Chaves et al, 2009). Dox, which is an anthracycline anti-neoplastic agent, is frequently used during the pre-operative and peri-operative periods, as it is often incorporated into the treatment of malignancies such as breast cancer, lymphoma, leukaemia, ovarian cancer, hepatocellular carcinoma and soft tissue sarcoma. Dox is reduced in the cellular cytoplasm by aldehyde and ketone reductases and detoxified, most probably by NADPH cytochrome P450 reductase catalyzed reduction of the oxygen-linked glycoside to deoxy-glycone forms (Di Fronzo et al, 1971 and Gutiérrez et al, 1983). Dox and its primary alcohol metabolite doxorubicinol are excreted unchanged in the bile and to a lesser extent in the urine. Accurate prediction of acute human clinical toxicity from Dox by empirical determination of plasma pharmacokinetics of Dox or its metabolites has not yet proved feasible (Brenner et al, 1984 and Ackland et al, 1989). However, some studies have suggested that Dox-induced toxicity correlates with deranged hepatic function as measured by bromo-sulfophthalein time, bilirubin, or indocyanine green clearance (Benjamin et al, 1974 and Brenner et al, 1984).

Recently, several polyphenolic antioxidants derived from grape seeds and skin has been implicated in cell protection (Cui et al, 2002). Grape seed proanthocyanidin have been demonstrated to exhibit a broad spectrum of pharmacological, therapeutic and chemoprotective properties. Grape seed Proanthocyanidin Extract (GSPE) demonstrates significant cytotoxicity towards human breast, lung and gastric adenocarcinoma cells (Bagchi et al, 2001). Epidemiological and experimental studies have revealed that mild to moderate drinking of wine, particularly red wine, attenuates the cardiovascular, cerebrovascular and peripheral vascular risks and kidney and liver diseases. Although the biochemical basis for such health benefits is not fully understood, this effect has been attributed to the alcohol-free portion containing antioxidants (Bagchi et al, 2002).

Vitamin E, a free-radical scavenger in the lipid compartments of cells and serum, is known for its beneficial antioxidant effects for a number of chronic diseases including cancer (Kline et al, 2004). Increased serum vitamin E levels have been reported to decrease lipid peroxidation, inhibit protein kinase C, 5-lipoxygenase, smooth muscle cell proliferation, platelet aggregation and oxygen burst in neutrophils and the oxygen burst in neutrophils (Brigelius-Flohe et al, 2002 and Peralta et al, 2006). Pre-treatment with vitamin E supplementation has been proven to show neuroprotective effect in patients treated with cisplatin (Pace et al, 2003). It has been reported to prevent several changes in serum enzymes and to protect increase in hematocrit, fall in leukocyte count, haemoglobin level, and mean osmotic fragility of erythrocytes (Sultana et al, 2006).

The present study was carried out to examine anti-oxidant potential of GSPE along with vitamin E on Dox induced oxidative stress. Since the free radical produced during the metabolism of the drug is considered to be responsible for alteration in various cellular enzyme activities, lipid peroxidation, antioxidant, and antioxidant enzymes, the effect of antioxidant GSPE, together with vitamin E, which intercepts the toxic free radicals was investigated in rats.

2. Material and Methods

2.1. Drugs and Chemicals

Proanthocyanidin (GSPE) and α-tocopherol- acetate was purchased from Sigma Chemicals, St Louis, MO, USA. All other chemicals were of analytical grade and solvents were of Qualigen grade, procured from local commercial sources.

2.2. Animal Model

Adult male rats of Wister strain weighing 200±50 gm were maintained under standard conditions of humidity, temperature (25±2 ºC) and light (12 h light/dark). They were fed standard rat pelleted diet obtained from Lipolin India and offered water ad libitum. Experimental animals were handled according to the Institutional legislation, regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

2.3. Experimental Design

Following the acclimatization period, the rats were randomly divided into five equal groups i.e. 10 rats in each group. Group I consisted of rats maintained on commercial rat chow diet throughout the experimental period and served as a control. Group II consisted of rats maintained on a commercial rat chow diet and treated with Dox intraperitoneally at a dose of 15 mg/kg to induce hepatotoxicity. Group III rats were maintained on a commercial rat chow diet and received GSPE orally at a dose of 150 mg/kg daily (Pataki et al, 2002) for 10 days before treatment with Dox. Group IV consisted of rats maintained on a commercial rat chow diet and treated with vitamin E orally at a dose of 400 IU/day (Folts, 2002) for 10 days prior to Dox administration. Group V consisted of rats maintained on a commercial rat chow diet with cotreatment with GSPE and vitamin E for10 days before injection with Dox.

At the end of experimental period the test animals were sacrificed by cervical decapitation under ether anaesthesia and liver was excised immediately and washed with ice-
cold saline. A homogenate (10%) of washed tissue (liver) was prepared in 0.01 M phosphate buffer (pH 7.4). The homogenate was centrifuged at 12000 rpm for 30 min; using high speed refrigerated centrifuge (Remi) at 4 ºC. The blood samples collected in plain centrifuge tube were kept in inclined position to allow complete clotting of blood and then centrifuge at 2500 rpm for 30 min. The resultant clear supernatant was pipetted out and preserved in small vials in the freezer at -20 ºC until assay for estimation of the following: 1) Total cholesterol was measured by an enzymatic method (Deeg & Ziegenhorn, 1983); 2) Low density lipoprotein cholesterol (LDL-c) was measured by a chemical method (Okada et al, 1998); 3) High density lipoprotein cholesterol (HDL-c) (Durrington, 1982); 4) triglycerides (Howdieshell et al, 1995); 5) aspartate aminotransferase (AST) and 6) alanine aminotransferase (ALT) (Smith & Taylor, 1972); 7) lactic dehydrogenase (LDH) (Gjerde & Mørland, 1985); 8) gamma glutamyl transferase (GGT) (Nandi & Chatterjee, 1988); 9) superoxide dismutase (SOD) (Lukaszewicz-Hussain & Moniuszko-Jakoniuk, 2004). Histopathological examination for apoptotic and necrotic cell death in the liver was also performed.

2.4. Statistical Analysis

The results were subjected to statistical analysis (SPSS Software Package) using Two-Way ANOVA. Values were considered significant at P<0.05.

3. Results

3.1. Biochemical Results

Biochemical Results are presented in Tables 1-3.

3.2. Histological Results

Figure 1a shows normal architecture of the liver in the control group. Histological examination of the liver revealed normal architecture of the hepatic lobules and hepatocytes. Animals treated with Dox showed marked accumulation of mononuclear cells (mononucleosis) in the portal tract (Figures 1b-1c), reflecting monocytic infiltration. It appeared that mononucleosis was stimulated by degeneration of hepatocytes, which was caused by Dox treatment. These degenerative changes are depicted in Figure 1d in the form of cytoplasmic eosinophilia and vacuolation, in addition to nuclear pyknosis of hepatocytes. Additionally, there were degenerative changes in the endothelial lining of hepatocytes in the form of endothelial and Kupffer cell nuclear pyknosis. Mononucleosis, which was widespread in the liver of Dox-treated animals, was observed to migrate from portal tract vessels into the surrounding connective tissue and reflecting stimulation of monocyte phagocytic system where mononuclear cells transformed into phagocytic cells.

In animals that received GSPE prior to administration of Dox, the normal histological pattern of the liver was essentially maintained. The hepatic cord, hepatocytes and hepatic sinusoids were normal (Figure 1e). Mild dilatation was observed in some of the hepatic sinusoids. This was more often observed in the central zone of some hepatic lobules.

In animals treated with vitamin E prior to Dox, most of the hepatocytes had a normal appearance, and only a few had a pyknotic nucleus (Figures 1f-1g). Furthermore, there was mild congestion of some of the hepatic sinusoids. This was a combined administration of vitamin E and GSPE prior

Table 1. Effects of Proanthocyanidin Extract and/or Vitamin E on the Serum Lipid Profile Level Among the Groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Total Cholesterol (mg/dl)</th>
<th>LDL-C (mg/dl)</th>
<th>HDL-C (mg/dl)</th>
<th>Triglycerides (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean±S.E.</td>
<td>P Value</td>
<td>Mean±S.E.</td>
<td>P Value</td>
</tr>
<tr>
<td><strong>Group I</strong></td>
<td>70.80±3.76</td>
<td>0.0001a</td>
<td>33.12±2.31</td>
<td>0.0001a</td>
</tr>
<tr>
<td><strong>Group II</strong></td>
<td>108.33±7.94</td>
<td>-</td>
<td>58.33±5.42</td>
<td>-</td>
</tr>
<tr>
<td><strong>Group III</strong></td>
<td>113.28±2.51</td>
<td>0.0001a</td>
<td>45.91±4.56</td>
<td>0.010a</td>
</tr>
<tr>
<td><strong>Group IV</strong></td>
<td>104.85±2.39</td>
<td>0.0001a</td>
<td>63.61±4.56</td>
<td>0.0001a</td>
</tr>
<tr>
<td><strong>Group V</strong></td>
<td>87.28±3.04</td>
<td>0.007a</td>
<td>57.62±3.41</td>
<td>0.0001a</td>
</tr>
</tbody>
</table>

*a Compared with the Control Group (P<0.05)
*b Compared with the Dox-Treated Group (P<0.05)
to Dox treatment (Figure 1h) appeared to have a prophylactic effect against the harmful effects of Dox. There were substantial differences in the effect of combined treatment compared with administration of either vitamin E or GSPE alone prior to treatment with Dox.

Table 2. Effects of GSPE and/or Vitamin E on Liver Function Tests in the Serum Among the Groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>AST (IU/L) Mean±S.E.</th>
<th>P Value</th>
<th>ALT (IU/L) Mean±S.E.</th>
<th>P Value</th>
<th>LDH (IU/L) Mean±S.E.</th>
<th>P Value</th>
<th>GGT (IU/L) Mean±S.E.</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>158.16±14.03</td>
<td>-</td>
<td>71.00±6.49</td>
<td>-</td>
<td>100.83±6.58</td>
<td>-</td>
<td>7.00±0.70</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>174.40±8.07</td>
<td>0.021*</td>
<td>108.60±3.20</td>
<td>0.020*</td>
<td>97.20±3.33</td>
<td>0.077</td>
<td>17.83±1.01</td>
<td>0.0001*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>202.42±17.07</td>
<td>0.024*</td>
<td>80.71±12.05</td>
<td>0.068</td>
<td>109.71±8.68</td>
<td>0.357</td>
<td>15.42±1.10</td>
<td>0.001*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>203.57±13.23</td>
<td>0.021*</td>
<td>79.85±4.64</td>
<td>0.061</td>
<td>121.71±8.19</td>
<td>0.036*</td>
<td>15.57±1.08</td>
<td>0.001*</td>
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<tr>
<td></td>
<td></td>
<td>149.42±6.93</td>
<td>0.041*</td>
<td>86.00±14.24</td>
<td>0.036*</td>
<td>109.57±2.76</td>
<td>0.365</td>
<td>16.42±1.06</td>
<td>0.001*</td>
</tr>
</tbody>
</table>

*Compared with the Control Group (P<0.05)
Compared with the Dox-Treated Group (P<0.05)

Table 3. Effects of Proanthocyanidin Extract and/or Vitamin E on Liver Tissue SOD, CAT and MDA Levels among the Groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>SOD (u/g) Mean±S.E.</th>
<th>P Value</th>
<th>CAT (u/g) Mean±S.E.</th>
<th>P Value</th>
<th>MDA (nmol/g) Mean±S.E.</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>39.36±0.46</td>
<td>0.032b</td>
<td>1.78±0.05</td>
<td>0.001b</td>
<td>10.23±0.47</td>
<td>0.002b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>23.00±9.75</td>
<td>-</td>
<td>1.33±0.04</td>
<td>-</td>
<td>12.56±0.22</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>41.54±2.32</td>
<td>0.747</td>
<td>1.99±0.04</td>
<td>0.067</td>
<td>12.65±0.46</td>
<td>0.001*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>41.78±0.90</td>
<td>0.721</td>
<td>2.08±0.04</td>
<td>0.015*</td>
<td>15.43±0.53</td>
<td>0.0001*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>39.64±2.44</td>
<td>0.968</td>
<td>1.97±0.04</td>
<td>0.097</td>
<td>15.07±0.08</td>
<td>0.001*</td>
</tr>
</tbody>
</table>

*Compared with the Control Group (P<0.05)
Compared with the Dox-Treated Group (P<0.05)

4. Discussion

Dox by virtue of its Quinone groups under aerobic conditions can undergo one-electron reduction to generate semi Quinone radical. Semi Quinone radical can rapidly react with O2 to form superoxide radical like hydroxyl radical that participates in the peroxidation of membrane lipids leading to increased malondialdehyde formation (Amudha et al, 2006). Generation within membrane and lipoproteins of peroxy and alkoxyl radicals, aldehydes and other products of lipid peroxidation affects liver substantially, causing formation of high molecular mass protein aggregates within the membrane, hence, increased level of malondialdehyde is an indicator of lipid peroxidation (Pryor, 1973). This is consistent with results where we observed hike in lipid peroxidation in liver in Dox administrated rats.

Our results established that intraperitoneal administration of Dox had profound effects on the liver as assessed by liver function tests and liver histology. In addition, Dox-induced apoptotic death as well as necrosis in the respective biotransformation is known to produce damaging free radicals in vivo (Ray et al, 2000). The liver processes most of the chemicals entering the body, and therefore, it has a high risk of damage. Hepatocytes (liver...
Parenchymal cells are the main functional cells of the liver (Ashwell & Harford, 1982).

Wang et al (2006) had suggested that Dox is rapidly eliminated from the circulation and delivered to the liver.

Figures 1 (a-h). Representative Rate Liver Section of Various Groups

Figure 1a represents rat liver section (100X) from group I, i.e. control animals, no pathological changes are present. Figures 1b-1c represents rat liver section (100X) from group II, i.e. Dox treated animals (15 mg/kg intraperitonial), marked accumulation of mononuclear cells (mononucleosis) in portal tract is seen. Figure 1d shows the degenerative changes in the form of cytoplasmic eosinophilia and vacuolation. Figure 1e represents rat liver section (100X) from group III, i.e. GSPE given prior to Dox administration (150 mg/kg daily, orally) for 10 days, the normal histological pattern of liver is maintained. Figures 1f-1g represents rat liver section (100X) from group IV, i.e. vitamin E was given prior to Dox, treatment (400 IU/day, orally) for 10 days, most of hepatocytes showed normal appearance, and a few had a pyknotic nucleus. Figure 1h represents liver section (100X) from group V, i.e. having combined treatment of GSPE a comes with vitamin E prior to Dox treatment, showed a prophylactic effect against the harmful effects of Dox.
via a sialoglycoprotein receptor (ASGPr) mediated mechanism. However, from the pharmacokinetic point of view, a rapid accumulation of drugs in the liver may not be beneficial (Levy, 1987). In addition, the rapid uptake of anti-cancer agents may induce liver damage due to the narrow therapeutic index of drug. The time that Dox is circulating in the blood may be essential to assure sustained interaction, yielding better tissue targeting and lower cytotoxicity (Takino et al, 1998). Our results also established that intraperitoneal administration of Dox had profound effects on the liver as assessed by liver function tests and liver histology.

Previous studies have suggested that oxLDL (oxidized low density lipoprotein) are produced as an intermediate agent during exacerbated oxidative stress, which contribute to various pathophysiological mechanisms underlying the process of liver fibrosis. Thus, an association exists between elevated oxLDL level and hepatocellular injury, in particular fibrosis (Karadeniz et al, 2008). High serum lipid levels in the present study (in group 2 (Dox. treated group), especially elevated levels of LDL-c, have been shown to be strongly related to the development of atherosclerosis. Atherosclerotic lesions are initiated via enhancement of LDL-c uptake by monocytes and macrophages. In the liver, uptake of plasma LDL-c is mediated via specific LDL-c receptors, but a scavenger receptor system is employed by macrophages (Choy et al, 2004).

Proanthocyanidins have been reported to possess a broad spectrum of pharmacological and medicinal properties against oxidative stress. GSPE has significantly better free radical scavenging ability than vitamin E and shows significant cytotoxicity towards gastric adenocarcinoma cells, while enhancing the growth of normal cells (Bagchi et al, 2000). GSPE provides protection against cancer chemotherapeutic drug-induced cytotoxicity in human liver cells by modulating cell cycle/apoptosis regulatory genes such as bcl-2, p53 and c-myc. In the previous study (Ray et al, 2000), pre-exposure of GSPE to Dox-induced heaptotoxicity provided almost complete protection of serum chemistry changes i.e. ALT and CPK (Creatine Phospho Kinase) and DNA damage. It also abolished apoptotic and necrotic cell death in liver cells. Histopathological examination of liver secretion demonstrated moderate to massive tissue damage. Proanthocyanidin shows excellent properties against oxidative stress. A previous study (Cetin et al, 2008) reported that grape seed extract protects the liver and inhibits methotrexate-induced oxidative stress.

In an attempt to ameliorate the chemotherapy associated cytotoxicity, we investigated the effect of GSPE on the liver. We found that GSPE administration prior to Dox treatment did not result in any changes in total cholesterol and triglycerides, but there was a significant (P<0.05) decrease in LDL-c while there was a significant (P<0.05) increase in HDL-c compared to Dox-treated rats and there was no change in AST, ALT, LDH and GGT levels compared with those in the Dox-treated group. Liver SOD and CAT levels were also increased while MDA levels did not change compared to Dox-treated group. Another study (Dulandu et al, 2007) showed that serum levels of AST, ALT, and LDH were significantly decreased with GSE treatment in bile ductligated rats. These results suggest that GSE protects the liver from oxidative damage following bile duct ligation in rats and this effect possibly involves the inhibition of neutrophil infiltration and lipid peroxidation, thus restoring oxidant and antioxidant activity.

This protection by GSPE may be linked to both inhibition of metabolism and/or detoxification of cytotoxic radicals. In addition, its presumed contribution to DNA repair may be another important attribute, which plays a role in its protective effects.

In the present study, there were no distinctive clinical signs, mortality or morbidity observed in any of the experimental groups during study period.

In the present study, oral administration of GPSE improved SOD and CAT levels. A previous study showed that GPSE administration reduced the levels of lipid peroxides and enhanced the antioxidant defence against reactive oxygen species produced under Dox treatment, thereby protecting liver cells (Chis et al, 2009).

With regard to vitamin E treatment, we found that there were no changes in the lipid profile, except for a significant increase in HDL-c levels compared with the Dox-treated group. There were also no changes in AST, ALT and GGT levels compared with those in the Dox-treated group. Liver SOD, CAT and MDA levels were significantly increased (P<0.05) compared with those in Dox-treated group. A previous study (Bagchi et al, 2000) demonstrated that GSPE provides significantly greater protection against free radicals and lipid peroxidation and DNA damage than vitamin E.

Combined treatment with proanthocyanidin and vitamin E significantly improved (P<0.05) the lipid profile by decreasing total cholesterol and triglycerides, and returning HDL-c levels to normal. Additionally, Liver function was significantly (P<0.05) decreased compared with that in the Dox-treated group, with a significant (P<0.05) increase in SOD, CAT and MDA levels compared with those in the Dox-treated group. It has been found that co-administration of α-tocopherol and Dox significantly minimizes lipid peroxide formation by Dox (Geetha et al, 1991). GSPE exhibits a dose-dependent inhibition of lipid peroxidation and DNA fragmentation in the liver and
brain. GSPE, with other anti-oxidants, provided significant protection against TPA (12-O-tetradecanoyl phorbol-13-acetate) induced oxidative damage (Bagchi et al, 1998). Histological examination of the liver shows similar patterns to those of serum chemistry changes and inhibition of both forms of cell death, i.e. necrosis and apoptosis (Bagchi et al, 2001). Our results suggested that administration of proanthocyanidin with vitamin E prior to Dox treatment results in better protection against drug-induced toxicity than vitamin E or GPSE alone.

5. Conclusion

The present investigation indicates that proanthocyanidin and vitamin E affect doxorubicin-induced hepatotoxicity by inhibiting free radical mediated tissue injury and lipid peroxidation, thus supporting antioxidants. It represents a potentially advantageous strategy with or prior to doxorubicin chemotherapy.

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