Protective effect of dl-α-lipoic acid on cyclophosphamide induced oxidative cardiac injury

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Abstract

Cyclophosphamide (CP), one of the most widely prescribed antineoplastic drugs could cause a lethal cardiotoxicity. The present study is aimed at evaluating the role of dl-α-lipoic acid (LA) in oxidative cardiac damage induced by CP. Adult male Wistar rats were divided into four treatment groups. Two groups received single intraperitoneal injection of CP (200 mg/kg BW) to induce cardiotoxicity, one of these groups received LA treatment (25 mg/kg BW for 10 days). A vehicle treated control group and a LA drug control were also included. Cardiotoxicity, evident from increased activities of serum creatine phosphokinase, lactate dehydrogenase, aspartate transaminase and alanine transaminase in CP administered rats, was reversed by LA treatment. CP administered rats showed abnormal levels of enzymic (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione-S-transferase) and non-enzymic antioxidants (glutathione, vitamin C and vitamin E) along with high malondialdehyde levels. However, normalized lipid peroxidation and antioxidant defenses were reported in the LA treated rats. These findings highlight the efficacy of LA as a cytoprotectant in CP induced cardiotoxicity.

Keywords: Cyclophosphamide; Cardiotoxicity; Lipoic acid; Lipid peroxidation; Antioxidants

1. Introduction

Cyclophosphamide (CP) is widely used as an anticancer and immunosuppressant drug. It is used for the treatment of chronic and acute leukemias, multiple myeloma, lymphomas, rheumatic arthritis and in preparation for bone marrow transplantation [1,2]. Although it has tumor selectivity, it also possesses a wide spectrum of toxicities [3]. The crucial factor for the therapeutic and toxic effects of CP is the requirement of metabolic activation by hepatic microsomal cytochrome P450 mixed functional oxidase system [4]. Administration of high doses of cyclophosphamide could cause a lethal cardiotoxicity, which presents as a
combination of symptoms and signs of myopericarditis leading to fatal complications such as congestive heart failure, arrhythmias, cardiac tamponade and myocardial depression [5]. The cardiotoxic effects of CP consist of acute, dose dependent cardiac damage, morphologically characterized by necrosis, hemorrhage and later development of fibrosis [2,6]. Reactive oxygen species have been implicated in the development of cardiotoxicity after CP administration [7]. Cardiotoxicity of cyclophosphamide may be controlled by pharmacological interventions that reduce oxidative stress.

Lipoic acid (LA) is a unique, effective and safe substance that displays the best possible scenario for natural antioxidant. It is referred to as universal antioxidant as it acts both in the membranous phase and aqueous phase [8]. It has specificity for free radical quenching, metal chelating activity and it also interacts and regenerates other cellular antioxidants [9]. We have also identified it to be cardioprotective in adriamycin toxicity [10]. The aim of the present study was to assess the oxidative status and the associated cardiac damage in CP administered rats. Further the impact of LA on myocardial damage was evaluated.

2. Materials and methods

2.1. Drugs and chemicals

Cyclophosphamide (Endoxan®) was purchased from German Remedies Limited, Goa, India. dl-α-Lipoic acid, 1,1,3,3-tetraethoxypropane and bovine serum albumin were procured from Sigma Chemicals, St. Louis, MO, USA. All other chemicals and solvents used were of highest purity and analytical grade.

2.2. Experimental protocol

Male albino rats of Wistar strain (140 ± 10 g) procured from Tamilnadu University for Veterinary and Animal Sciences, Chennai, India were used for the study. Animals were fed with commercially available standard rat pelleted feed (M/s Pranav Agro Industries Ltd., India) under the trade name Amrut rat/mice feed and water was provided ad libitum. The rats were housed under conditions of controlled temperature (25 ± 2°C) and acclimatized to 12:12 h light/dark cycle. Animal experiments were conducted according to the guidelines of institutional animal ethical committee.

Rats were divided into four groups, each consisting of six animals. Group I served as the vehicle treated controls. Group II animals were injected intraperitoneally with a single dose of CP (200 mg/kg BW) dissolved in saline, on the first day of the experimental period. Group III animals received LA (25 mg/kg BW, orally) dissolved in saline at alkaline pH (7.8) daily for 10 days. In Group IV, animals were administered CP as in Group II, immediately followed by administration of LA daily for 10 days.

After the 10 days experimental period, all the animals were anesthetized and decapitated. Heart tissues were immediately excised and rinsed in ice cold physiological saline. The tissues were homogenized in 0.01 M Tris-HCl buffer (pH 7.4) and aliquots of this homogenate were used for the assays. Blood was collected and serum was separated for analysis of biochemical parameters.

2.3. Enzymatic indices of cellular damage

Lactate dehydrogenase (LDH) was assayed by the method of King [11]. The method is based on the ability of LDH to form pyruvate in the presence of coenzyme NAD+. The pyruvate formed was made to react with 2,4-dinitrophenylhydrazine in hydrochloric acid. The hydrazone formed turns into an orange coloured complex in alkaline medium, which was measured at 420 nm. Aspartate transaminase (AST) and alanine transaminase (ALT) were estimated by the method of King [12]. Their activities were expressed in terms of μmoles of pyruvate liberated/min/mg of protein. The activity of creatine phosphokinase (CPK) was assayed by the method of Okinaka et al. [13] and was expressed as μmoles of phosphorus liberated/min/mg protein. Protein content was estimated by the method of Lowry et al. [14].

2.4. Lipid peroxidation

Lipid peroxidation (LPO) was determined by the method of Hogberg et al. [15]. Malondialdehyde (MDA), formed as an end product of the peroxidation of lipids, served as an index of the intensity of oxidative stress. MDA reacts with thiobarbituric acid to generate a coloured product which absorbs at 532 nm. The fer-
rous sulphate and ascorbate induced lipid peroxidation system contained 10 mM ferrous sulphate and 0.2 mM ascorbate as inducers [16].

2.5. Enzymic antioxidants

Superoxide dismutase (SOD) was assayed according to the method of Marklund and Marklund [17]. The degree of inhibition of the autoxidation of pyrogallol at an alkaline pH by SOD was used as a measure of the enzyme activity. Glutathione peroxidase (GPx) activity was assessed in terms of utilization of glutathione [18]. It is based on the reaction between glutathione remaining after the action of GPx, and 5,5-dithio-bis(2-nitrobenzoic acid) resulting in a complex that absorbs maximally at 421 nm. Catalase (CAT) was assayed by the method of Sinha [19]. In this method, dichromate in acetic acid was reduced to chromic acetate when heated in the presence of hydrogen peroxide (H₂O₂), with the formation of perichoric acid as an unstable intermediate. Chromic acetate thus produced was measured colorimetrically at 610 nm. Glutathione reductase (GR) that utilizes NADPH to convert oxidised glutathione (GSSG) to the reduced form was assayed by the method of Staal et al. [20]. Glutathione-S-transferase (GST) was assayed by the method of Habig et al. [21].

2.6. Non-enzymic antioxidants

Total reduced glutathione (GSH) was determined by the method of Moron et al. [22]. Vitamin C was assayed by the procedure of Omaye et al. [23]. Vitamin C was oxidized by copper to form dehydroascorbic acid and diketoglutaric acid, which were treated with 2,4-dinitrophenylhydrazine to form the derivative of bis-2,4-dinitrophenylhydrazine. This compound in strong sulphuric acid undergoes rearrangements to form a product that is measured at 520 nm. Vitamin E was estimated by the method of Desai et al. [24].

2.7. Statistical analysis

The results were expressed as mean ± standard deviation (S.D.) for six animals in each group. Differences between groups were assessed by one-way analysis of variance (ANOVA) using the SPSS software package for Windows. Post hoc testing was performed for inter-group comparisons using the least significance difference (LSD) test; significance at P-values <0.001, <0.01, <0.05 have been given respective symbols in the tables.

3. Results

Intraperitoneal administration of a single dose of CP (200 mg/kg BW) induced severe biochemical changes as well as oxidative damage in cardiac tissue. Table 1 shows the abnormally elevated activities of serum enzymes that indicate cellular damage caused by CP. The activities of serum CPK, LDH, AST and ALT increased by 86, 75.3, 53.42 and 60%, respectively, in Group II animals when compared with control. Activities of these marker enzymes were restored to near normalcy (P < 0.001) after LA administration.

The heart tissue of CP treated rats (Group II) showed a 2.49-fold increase in basal LPO as well as a 1.86- and 1.74-fold increase in LPO in the presence of inducers such as ascorbate and ferrous sulphate respectively, when compared with the control. Activities of these marker enzymes were restored to near normalcy (P < 0.001) after LA administration.

Table 1

<table>
<thead>
<tr>
<th>Serum parameters</th>
<th>Group I (control)</th>
<th>Group II (CP)</th>
<th>Group III (LA)</th>
<th>Group IV (CP + LA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPK</td>
<td>8.32 ± 0.64</td>
<td>15.12 ± 1.58***</td>
<td>8.17 ± 0.61</td>
<td>9.05 ± 0.66</td>
</tr>
<tr>
<td>LDH</td>
<td>6.02 ± 0.63</td>
<td>10.55 ± 1.30***</td>
<td>6.12 ± 0.66</td>
<td>7.06 ± 0.67***</td>
</tr>
<tr>
<td>AST</td>
<td>0.73 ± 0.06</td>
<td>1.12 ± 0.13***</td>
<td>0.77 ± 0.08</td>
<td>0.83 ± 0.07***</td>
</tr>
<tr>
<td>ALT</td>
<td>0.50 ± 0.04</td>
<td>0.80 ± 0.09***</td>
<td>0.51 ± 0.05</td>
<td>0.56 ± 0.05***</td>
</tr>
</tbody>
</table>

Results are given as mean ± S.D. for six rats. Units—CPK: μmol per min/mg protein; LDH: μmol per min/mg protein; AST and ALT: μmol per min/mg protein. Comparisons are made between:

a—Group I and Group II, III, IV; b—Group II and Group IV. The symbols (***) and (*) represent statistical significance at P < 0.001 and P < 0.05, respectively.
Table 2: Effect of cyclophosphamide and lipoic acid on cardiac lipid peroxidation

<table>
<thead>
<tr>
<th>Lipid peroxidation</th>
<th>Group I (control)</th>
<th>Group II (CP)</th>
<th>Group III (LA)</th>
<th>Group IV (CP + LA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>1.46 ± 0.16</td>
<td>3.64 ± 0.23***</td>
<td>1.43 ± 0.12</td>
<td>1.58 ± 0.16***</td>
</tr>
<tr>
<td>Ferric sulfate induced</td>
<td>5.15 ± 0.51</td>
<td>8.98 ± 0.84***</td>
<td>5.28 ± 0.56</td>
<td>5.85 ± 0.53***</td>
</tr>
<tr>
<td>Ascorbate induced</td>
<td>3.02 ± 0.35</td>
<td>5.63 ± 0.61***</td>
<td>3.25 ± 0.37</td>
<td>3.92 ± 0.38***</td>
</tr>
</tbody>
</table>

Results are given as mean ± S.D. for six rats. Units: nmoles of MDA formed/min/mg protein. Comparisons are made between: a—Group I and Group II, III, IV; b—Group II and Group IV. The symbols (***) and (**) represent statistical significance at \( P < 0.001 \) and \( P < 0.01 \), respectively.

Table 3: Effect of cyclophosphamide and lipoic acid on the activities of cardiac enzymic antioxidants

<table>
<thead>
<tr>
<th>Antioxidant enzymes</th>
<th>Group I (control)</th>
<th>Group II (CP)</th>
<th>Group III (LA)</th>
<th>Group IV (CP + LA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD</td>
<td>2.00 ± 0.18</td>
<td>1.32 ± 0.13***</td>
<td>2.00 ± 0.23</td>
<td>1.92 ± 0.24***</td>
</tr>
<tr>
<td>CAT</td>
<td>60.67 ± 5.82</td>
<td>38.03 ± 3.37***</td>
<td>61.83 ± 6.68</td>
<td>56.83 ± 5.78***</td>
</tr>
<tr>
<td>GPx</td>
<td>6.82 ± 0.57</td>
<td>4.18 ± 0.49***</td>
<td>6.90 ± 0.62</td>
<td>6.28 ± 0.66***</td>
</tr>
<tr>
<td>GR</td>
<td>1.22 ± 0.12</td>
<td>0.59 ± 0.06***</td>
<td>1.19 ± 0.13</td>
<td>1.08 ± 0.10***</td>
</tr>
<tr>
<td>GST</td>
<td>1.13 ± 0.15</td>
<td>0.74 ± 0.08***</td>
<td>1.15 ± 0.15</td>
<td>1.00 ± 0.13***</td>
</tr>
</tbody>
</table>

Results are given as mean ± S.D. for six rats. Units of enzyme activity: SOD: Units/mg protein, one unit is equal to the amount of enzyme that inhibits the autooxidation reaction by 50%; Catalase: μmoles of H₂O₂ consumed/min/mg protein; GPx: μg of GSH consumed/min/mg protein; GR: nmoles of NADPH oxidized/min/mg protein; GST: nmoles of CDNB–GSH conjugate formed/min/mg protein. Comparisons are made between: a—Group I and Group II, III, IV; b—Group II and Group IV. The symbols (***) and (**) represent statistical significance at \( P < 0.001 \), \( P < 0.01 \), respectively.

Table 4: Effect of cyclophosphamide and lipoic acid on the cardiac non-enzymatic antioxidants

<table>
<thead>
<tr>
<th>Antioxidants</th>
<th>Group I (control)</th>
<th>Group II (CP)</th>
<th>Group III (LA)</th>
<th>Group IV (CP + LA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH</td>
<td>6.12 ± 0.54</td>
<td>3.71 ± 0.28***</td>
<td>6.52 ± 0.40</td>
<td>5.92 ± 0.64***</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>1.26 ± 0.16</td>
<td>0.81 ± 0.08***</td>
<td>1.35 ± 0.11</td>
<td>1.11 ± 0.15***</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>1.03 ± 0.12</td>
<td>0.77 ± 0.08***</td>
<td>1.11 ± 0.10</td>
<td>0.94 ± 0.08***</td>
</tr>
</tbody>
</table>

Results are given as mean ± S.D. for six rats. Units: GSH, vitamin C and vitamin E: μg/mg protein. Comparisons are made between: a—Group I and Group II, III, IV; b—Group II and Group IV. The symbols (***) and (**) represent statistical significance at \( P < 0.001 \) and \( P < 0.01 \), respectively.

Table 3 presents the activities of antioxidant enzymes. Statistically significant (\( P < 0.001 \)) decrease in the activities of antioxidant enzymes (SOD, GPx, CAT, GR and GST) was observed in Group II. These changes highlight the deteriorating antioxidant status in the CP group. LA administration elevated the activities of SOD, CAT, GPx, GR and GST by 1.45-, 1.46-, 1.5-, 1.83- and 1.35-fold, respectively, in Group IV when compared to CP induced Group II animals.

A marked decline (\( P < 0.001 \)) in the levels of non-enzymic antioxidants GSH, vitamin C and vitamin E was noted in CP treated rats (Table 4). Significant increase (\( P < 0.001; \ P < 0.01 \) in the levels of non-enzymic antioxidants was prominent in the lipoic acid treated group. The restoration of enzymic and non-enzymic antioxidant levels towards control range by LA indicates its protective effect against oxidative stress induced by CP.

4. Discussion

High doses of CP can cause an acute form of cardiotoxicity within 10 days of its administration [25]. Administration of intermittent massive dosage of CP has been found to be advantageous in chemotherapy [26]. Evidence of cardiomyopathy with vascular involvement in rats has been reported by Hopkins et al. [27]. Cellular mechanisms of cardiotoxicity are thought to be mediated by an increase in free oxygen radicals through intracellular phosphoramide mustard, the principal alkylating metabolite of CP which affects en-
dothelium and ion transport mechanisms [7]. Cardiac pathology from CP is by direct endothelial damage with extravasation of proteinaceous fluid, high concentrations of CP and erythrocytes into the myocardial interstitium and muscle cells [3,28]. The determination of antioxidant status of heart after CP administration in experimental models is important to develop strategies to reduce cardiotoxicity.

In the present study, CP administration significantly increased the activities of serum CPK, LDH, AST and ALT. These observations are consistent with previous reports [29,30]. Increased activities of these enzymes in serum are well known diagnostic indicators of cardiac injury. During myocardial necrosis these enzymes are released from heart into the blood stream [31]. LA restored the activities of these marker enzymes to near normalcy. This suggests the cardioprotective role of LA, which is in line with a recent report where LA has been shown to be protective against cardiac injury elicited by ischemia-reperfusion [32].

Free radicals cause membrane injury by initiating LPO which results in loss of function and integrity of myocardial membranes. The present data reveal that CP exposure produced a marked oxidative impact as evidenced by increased LPO. This might result from increased production of free radicals and/or a decrease in antioxidant status. As LA is soluble in both membranous and aqueous phases, it effectively prevents the damage of cell membranes by lipid peroxides.

The myocardium has a variety of endogenous antioxidants. The major antioxidant enzymes SOD, CAT and GPx act in coordination and provide cellular defense against reactive oxygen species (ROS). Decline in the activities of these enzymes after CP administration might be due to inactivation of these enzymes by ROS. This decline further aggravates the levels of free radicals in heart. GPx and CAT protect SOD against inactivation by H$_2$O$_2$. Reciprocally SOD may protect CAT and GPx from inhibition by superoxide radicals [33]. The low levels of enzymic antioxidants and GSH in heart make it vulnerable to free radical damage [34]. LA scavenges hydroxyl radicals, hypochlorous acid, nitric oxide, peroxynitrite, H$_2$O$_2$ and singlet oxygen while dihydroxyacid (DHLA) scavenges superoxide radical and peroxyl radicals thereby preventing the free radical mediated inactivation of enzymes, restoring them to normalcy [9]. Besides, another possible reason for the lowered antioxidant activities in the CP challenged tissues may be the unit expression of enzyme activity. The specific activity of the enzymes is expressed as its activity relative to the total protein content. Since CP induces fibrosis and protein efflux into the heart, it further exaggerates the already down regulated antioxidant system. LA improves endothelial function [35], which in turn reduces protein transudation and therefore enhances the specific activity of antioxidant enzymes.

In the present study, a significant decline in GSH levels was prominently noted in CP induced group. Low cardiac glutathione levels is a risk factor for developing CP induced congestive heart failure [36]. The GSSG/GSH ratio is one of the most important parameters characterizing the prooxidant/antioxidant balance and its increase indicates the presence of oxidative damage. The role of GSH in repairing oxidative injury of cells and thereby protecting cardiac myocytes after 4-hydroxycyclophosphamide administration has been reported [37]. The redox system, LA/DHLA can regenerate the glutathione system. Exogenously supplied lipoate is rapidly taken up by a variety of tissues and can be reduced to the powerful antioxidant DHLA. Cysteine availability is the rate-limiting factor in GSH biosynthesis. LA/DHLA system induces the cystine/cysteine uptake and thereby increases GSH synthesis [38].

Inhibition of GSH metabolizing enzymes, GR and GST was noted after CP administration. GSSG formed from the reaction of GPx is subsequently reduced back to GSH at the expense of NADPH by GR. GR contains one or more sulphydryl group residues, which are essential for the catalytic activity and are vulnerable to free radicals [39]. LA supplementation enhanced GR activity indirectly regenerating the glutathione pool. GST, a phase 2 enzyme detoxifies electrophilic species via an enzyme catalysed conjugation reaction and also plays an active role in detoxification of cytotoxic lipid peroxidation [21,40]. The decreased activity of GST observed in our study may be partly due to the lack of its substrate (GSH) and also because of oxidative modification of its protein structure.

The protective actions of enzymic antioxidants are supported by antioxidant vitamins, which were found to be depleted after CP administration. Vitamin C acts as the first line of antioxidative defense in the aqueous compartment while vitamin E is a chain breaking antioxidant present in biological membranes. LA
affords protection by recycling ascorbate which in turn recycles the membrane antioxidant vitamin E [8]. LA causes a significant induction of key cellular antioxidants and phase 2 enzymes in cardiomyocytes to counter oxidative injury [41].

From these observations it is possible to conclude that CP administration results in pronounced oxidative stress and myocardial damage. LA was found to be effective in normalizing the antioxidants as well as the cardiac markers. Further studies are warranted into the role and mechanism of this readily available dietary supplement in CP induced oxidative damage of heart.

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