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Protective effect of DL-α-lipoic acid on cyclophosphamide induced hyperlipidemic cardiomyopathy

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Abstract

Cyclophosphamide is a potent alkylating agent used in cancer chemotherapy and immunosuppression. The present study is aimed at evaluating the role of a potent antioxidant lipoic acid in cyclophosphamide induced hyperlipidemic cardiomyopathy. Adult male Wistar rats were divided into four treatment groups. Two groups received single intraperitoneal injection of cyclophosphamide (200 mg/kg body weight) to induce cardiotoxicity, one of these groups received lipoic acid treatment (25 mg/kg body weight, orally for 10 days). A vehicle treated control group and a lipoic acid drug control were also included. Cyclophosphamide administration resulted in abnormal elevation of serum lipids. Similarly in the cardiac tissue, the levels of free cholesterol, esterified cholesterol, triglycerides were increased significantly ($P<0.05$) while the levels of phospholipids and free fatty acids were reduced significantly unlike serum ($P<0.05$). Serum Low Density Lipoprotein (LDL) and Very Low Density Lipoprotein (VLDL) cholesterol increased significantly ($P<0.05$) while High Density Lipoprotein (HDL) cholesterol ($P<0.05$) decreased significantly when compared to controls. These changes corroborated with the abnormal distortion in the activities of lipid metabolizing enzymes in cyclophosphamide treated group. Supplementation of lipoic acid reverted these abnormalities in the lipid levels and activities of lipid metabolizing enzymes to near normalcy after cyclophosphamide administration.

Keywords: Cyclophosphamide; Hyperlipidemia; Serum; Heart; Lipoic acid

1. Introduction

Cyclophosphamide is a widely used alkylating agent. Since its original production, the clinical use of cyclophosphamide has been extended from neoplastic diseases to organ transplantation and diverse disorders wherein it is used as an immunosuppressive agent. It is used for the treatment of chronic and acute leukemias, multiple myeloma, lymphomas, rheumatic arthritis and in preparation for bone marrow transplantation (Dollery, 1999; Goldberg et al., 1986). Cyclophosphamide is a prodrug activated by liver microsomal cytochrome P450 mixed function oxidase (Struck et al., 1984). Although it has tumor selectivity, it also possesses a wide spectrum of toxicities (Fraiser et al., 1991). Acute cardiotoxicity such as cardiac decompensation and cardiomyopathy has been associated with high dose cyclophosphamide therapy. The pathogenesis of acute cardiotoxicity may be attributed to an increase in free oxygen radicals in oxazaphosphorine induced cardiotoxicity (Schimmel et al., 2004). Hypercholesterolemia, hypertriglyceridemia and impaired secretion of heart lipoprotein lipase have been reported in cyclophosphamide treated rabbits (Loudet et al., 1984; Lespine et al., 1997). Perturbation of energy and lipid homeostasis plays a central role in the pathogenesis of heart disease. Primary and/or secondary alterations of lipid metabolism pathways in various conditions lead to myocardial lipid accumulation and lipotoxic cardiomyopathy. Defects in fatty acid uptake, oxidation and secretion can disrupt myocardial energy and lipid homeostasis (Yang and Cheng, 2005). Treatment of cultured vascular smooth muscle cells with free radicals resulted in cholesterol accumulation (Gesquiere et al., 1999). Antioxidants have been reported to play a beneficial role in the reduction of cardiovascular diseases.

Lipoic acid is an ideal antioxidant that directly quenches free radicals, inhibits reactive oxygen-generators and regenerates other

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Table 1
Effect of cyclophosphamide and lipoic acid on serum lipids

Parameter (mg/dl serum)	Group I (control)	Group II (cyclophosphamide)	Group III (lipoic acid)	Group IV (cyclophosphamide+lipoic acid)
Free cholesterol	38.17±4.71	61.67±5.20[a]	37.33±3.78	40.83±5.19[b]
Esterified cholesterol	49.50±4.37	78.17±5.95[a]	49.83±4.71	54.17±4.62[b]
Phospholipid	101.33±8.52	148.67±11.33[a]	101.02±8.35	104.07±9.51[b]
Free fatty acid	13.85±1.71	28.83±2.14[a]	13.67±1.37	16.58±1.80[a,b]
Triglyceride	67.33±6.25	104.50±9.16[a]	67.67±7.28	74.67±6.09[b]

Results are given as mean±S.D. for six rats. Comparisons are made between: [a] — group I and groups II, III, IV; [b] — group II and group IV. Values are statistically significant at $P<0.05$.

antioxidants (Packer et al., 1995). The marked effects of lipoic acid on risk factors for cardiovascular disease, both lipid and hemostatic, are of particular importance because they indicate potential antithrombotic and antiatherosclerotic actions that could prove beneficial in cardiac diseases and merit further study (Ford et al., 2001). The present study is aimed at evaluating the role of lipoic acid in cardiac lipemic derangements in cyclophosphamide administered rats.

2. Materials and methods

2.1. Drugs and chemicals

Cyclophosphamide (Endoxan®) was purchased from German Remedies Limited, Goa, India. DL-α-Lipoic acid 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 cyclophosphamide (200 mg/kg body weight) dissolved in saline, on the first day of the experimental period. Group III animals received lipoic acid (25 mg/kg body weight, orally) dissolved in saline at alkaline pH (7.8) daily for 10 days. In group IV, animals were administered cyclophosphamide as in group II, immediately followed by administration of lipoic acid 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 plasma and serum were separated for analysis of biochemical parameters.

2.3. Lipid profile

Lipids were extracted from the cardiac tissue according to the method of Folch et al. (1957) using chloroform–methanol (2:1 v/v). Cholesterol was estimated by the method of Parekh and Jung (1970) using ferric acetate–uranyl acetate as the chromogenic reagent. The free cholesterol was precipitated as its digitonide according to the method of Sperry and Webb (1950) and cholesterol in the precipitate was estimated. The esterified cholesterol was arrived at from the difference between the total and free cholesterol values. Phospholipids were determined by the method of Rouser et al. (1970). Serum triglycerol and free fatty acid were quantitated colorimetrically (Rice, 1970; Hron and Menahan, 1981).

2.4. Lipoproteins

Serum lipoproteins: Low Density Lipoprotein (LDL), Very Low Density Lipoprotein (VLDL) and High Density Lipoprotein

Table 2
Effect of cyclophosphamide and lipoic acid on cardiac lipid status

Parameter (mg/g wet tissue)	Group I (control)	Group II (cyclophosphamide)	Group III (lipoic acid)	Group IV (cyclophosphamide+lipoic acid)
Free cholesterol	3.45±0.42	4.22±0.46[a]	3.40±0.39	3.55±0.40[b]
Esterified cholesterol	1.82±0.26	2.85±0.39[a]	1.80±0.22	1.92±0.28[b]
Phospholipid	13.83±1.47	9.50±1.87[a]	14.02±1.67	12.98±1.82[b]
Free fatty acid	2.98±0.23	1.98±0.25[a]	3.02±0.19	2.85±0.25[b]
Triglyceride	4.53±0.53	7.43±0.96[a]	4.55±0.46	4.82±0.35[b]

Results are given as mean±S.D. for six rats. Comparisons are made between: [a] — group I and groups II, III, IV; [b] — group II and group IV. Values are statistically significant at $P<0.05$.

Table 3
Effect of cyclophosphamide and lipoic acid on serum lipoprotein fractions

Parameter (mg/dl serum)	Group I (control)	Group II (cyclophosphamide)	Group III (lipoic acid)	Group IV (cyclophosphamide+lipoic acid)
LDL cholesterol	12.67±1.51	28.33±2.58[a]	12.58±1.02	14.17±1.72[b]
HDL cholesterol	41.83±3.82	28.83±3.97[a]	42.02±3.74	40.17±3.13[b]
VLDL cholesterol	31.42±3.44	46.67±3.56[a]	31.50±3.08	35.83±3.31[a,b]

Results are given as mean±S.D. for six rats. Comparisons are made between: [a] — group I and groups II, III, IV; [b] — group II and group IV. Values are statistically significant at $P<0.05$.

(HDL) were fractionated by a dual precipitation technique (Wilson and Spiger, 1973). After the fractional precipitation, the cholesterol content of each fraction was estimated as mentioned earlier. Values are expressed as mg/dl of serum.

2.5. Lipid metabolizing enzymes

Cholesterol ester hydrolase (CEH) in heart was assayed by the method of Kothari et al. (1970) with slight modification of Kritchevsky and Kothari (1973). The free cholesterol liberated from cholesterol oleate was precipitated. The precipitate was processed and then dissolved in 3 ml of uranyl acetate and the cholesterol content was estimated as described earlier. Cholesterol ester synthetase (CES) in heart was estimated by the method of Kothari et al. (1973). The cholesterol digitonide, which was precipitated after centrifugation, was washed twice with acetone–ether mixture and finally with ether. The cholesterol content was estimated as mentioned earlier. The activity of lipoprotein lipase (LPL) was assayed in the cardiac tissue by the method of Schmidt (2000). The colour developed was read at 430 nm. LPL activity was expressed as μmol of free fatty acids liberated/h/mg protein. Plasma lecithin: cholesterol acyl trasferase (LCAT) activity was assayed by the method of Legrand et al. (1979) with modifications of Hitz et al. (1983). The colour developed was read at 540 nm. LCAT activity was expressed as nmol of cholesterol esterified/h/mg protein. Protein content was estimated by the procedure of Lowry et al. (1951).

2.6. 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; $P<0.05$ was considered significant for all comparisons.

3. Results

Intraperitoneal administration of cyclophosphamide resulted in a significant increase in the levels of serum lipids. The lipid levels in serum of cyclophosphamide treated rats were significantly increased ($P<0.05$) when compared to controls (Table 1). Supplementation of lipoic acid to group IV animals prevented the increase in serum lipid levels highlighting its hypolipidemic role.

Table 2 shows the effects of cyclophosphamide and lipoic acid on lipid fractions in the heart tissue. Cyclophosphamide treated group II animals showed a significant increase in the levels of free cholesterol ($P<0.05$), the storage form of cholesterol, esterified cholesterol ($P<0.05$) and triglycerides ($P<0.05$). In contrast a significant decrease in the levels of free fatty acids ($P<0.05$) and phospholipids ($P<0.05$) was observed in group II animals. Lipoic acid counteracted these abnormal cardiac changes and restored the levels of cardiac lipid levels to near normalcy.

Table 3 presents the serum lipoprotein-cholesterol profile of LDL, VLDL and HDL. A marked decrease in HDL cholesterol ($P<0.05$) along with a 2.23 fold increase in LDL and 1.48 fold increase in VLDL was noted in cyclophosphamide treated rats. Lipoic acid significantly increased the HDL ($P<0.05$) and decreased the levels of LDL ($P<0.05$) and VLDL cholesterol ($P<0.05$) levels when compared to group II cyclophosphamide administered rats.

The distortion in lipid levels corroborated with abnormalities in the activities of lipid metabolizing enzymes in cyclophosphamide group. Hypercholesterolemic changes in these rats maybe explained by a marked reduction in the activities of fat splitting enzymes, such as plasma LCAT and cardiac LPL by 33.37% and 35.56% respectively, when compared to controls (Table 4). The

Table 4
Effect of cyclophosphamide and lipoic acid on the activities of lipid metabolizing enzymes

Parameter	Group I (control)	Group II (cyclophosphamide)	Group III (lipoic acid)	Group IV (cyclophosphamide+lipoic acid)
Heart				
CES	9.05±0.84	14.25±1.72[a]	9.03±1.17	10.58±1.02[a,b]
CEH	15.08±1.50	11.08±1.43[a]	15.02±1.41	14.08±1.86[b]
LPL	13.92±1.43	8.97±1.28[a]	13.27±1.18	12.90±1.91[b]
Plasma				
LCAT	8.18±0.58	5.45±0.54[a]	8.22±0.75	7.67±0.79[b]

Results are given as mean±S.D. for six rats. Units of enzyme activity: CES: nmol of cholesterol esterified/h/mg protein at 37 °C; CEH: nmol of cholesterol liberated/h/mg protein at 37 °C; LPL: μmol of free fatty acids liberated/h/mg protein at 37 °C; LCAT: μmol of cholesterol esterified/h/mg protein at 37 °C. Comparisons are made between: [a] — group I and groups II, III, IV; [b] — group II and group IV. Values are statistically significant at $P<0.05$.

activity of CES increased by 57.46% and simultaneously the activity of CEH decreased by 26.52% in the cardiac tissue of cyclophosphamide treated rats when compared to controls. Lipoic acid restored the activities of these lipid metabolizing enzymes to near normalcy.

4. Discussion

The metabolism and physiology of lipids and lipoproteins is a dynamic integrated process. Lipoprotein abnormalities resulting in the disruption of serum and cellular lipid levels account for the genesis of vascular diseases. The acrolein-lysine adducts detected in the aorta and plasma LDL of cyclophosphamide treated animals suggest that these adducts wherein acrolein is a metabolite of cyclophosphamide, may play a role in the development of atherosclerosis or atherogenesis (Arikketh et al., 2004). Cyclophosphamide is known to result in hypertriglyceridemia and hypercholesterolemia, which are well known risk factors in cardiovascular diseases (Loudet et al., 1984).

Cyclophosphamide induced elevation in cholesterol levels could be due to increase in biosynthesis and decrease in its utilization. Cyclophosphamide induces free radicals (Lee et al., 1996), which may cause cellular cholesterol accumulation, (a) by increasing cholesterol biosynthesis and its esterification, (b) by decreasing cholesteryl ester hydrolysis and (c) by reducing cholesterol efflux (Gesquiere et al., 1999). The conversion of cholesterol to bile acids is quantitatively the most important mechanism for degradation of cholesterol. However, McClure and Stupans (1992) previously reported that after 7 days following a single dose of cyclophosphamide (200 mg/kg body weight) there was a decrease in cytochrome P450 activity in male rats, which may in turn depress cholesterol 7-hydroxylase activity, the key enzyme in the conversion of cholesterol to bile acids. Decline in the cardiac phospholipid content with a concomitant increase in the serum could be due to the peroxidation of unsaturated membrane lipids by free radicals in biomembranes and tissues causing the leakage of these lipids into circulation (Muralikrishnan et al., 2001).

Cholesterol and phosholipids are carried in plasma by lipoproteins, which are synthesized and secreted by the intestine and liver. VLDL and HDL are secreted from the liver into the bloodstream. Boren et al. (1998) have indicated that cardiac apoB enables the heart to secrete excess lipid in lipoproteins. In plasma, VLDL is degraded into IDL and LDL by the action of the enzyme LPL and through the exchange reactions with HDL. LDL serves as a major carrier of cholesterol to extrahepatic tissues. High levels of LDL are associated with an increased risk of cardiovascular disease whereas high levels of HDL afford protection by reverse cholesterol transport to liver. Previously in cyclophosphamide treated rats, lipid composition showed that HDL cholesterol was very low comparatively to a high VLDL cholesterol (Loudet et al., 1984). In these animals VLDL was larger than normal, corresponding to triglyceride enrichment (Lespine et al., 1988).

Triacylglycerols are degraded by the LPL to fatty acids, which are the chief sources of energy. LPL is predominantly present in the skeletal muscle, cardiac muscle and adipose tissue. Defective secretion of LPL may contribute to the poor expression of lipolytic activity in the vascular bed and to the occurrence of hypertriglyceridemia during cyclophosphamide treatment. Simultaneously heart LPL activity was also decreased in fasted animals (Lespine et al., 1997). Due to the alterations in LPL activity, increase in triglycerides was associated with a drop in fatty acid levels in heart of group II cyclophosphamide treated rats. The moderate increase in the rate of triacylglycerol synthesis by the liver contributes to the occurrence of hypertriglyceridaemia in cyclophosphamide treated rats (Lespine et al., 1993). Hypercholesterolemic changes in these rats maybe explained by a marked reduction in the activities of fat splitting enzymes, such as plasma LCAT and cardiac LPL. LCAT is secreted by the hepatocytes and released into the plasma. It converts cholesterol into long chain cholesteryl ester on HDL and favours reverse cholesterol transport from tissues to liver. The esterification of cholesterol by LCAT leads to the remodeling of the lipoprotein HDL and results in the formation of large HDL particles that are known to offer protection against coronary artery disease (Subramanian et al., 2003). Cholesterol esterification in the tissue is reported to be mediated through CES (Proudlock and Day, 1972). The activity of CEH may be reduced due to excessive increase in CES.

Antioxidants such as lipoic acid could be beneficial in countering cholesterol accumulation by scavenging free radicals. Lipoic acid is a universal antioxidant that acts in both membranous phase and aqueous phase (Kagan et al., 1992). Exogenously supplemented lipoic acid is reduced to the powerful antioxidant dihidrolipoic acid. Lipoic acid was effective in preventing cardiac lipid peroxidation induced by cyclophosphamide (Mythili et al., 2004). Hypolipidemic effect of lipoic acid has been noted when treatment with lipoic acid starting with the high cholesterol diet, limited the diet-dependent increase of lipids in the plasma, liver, and aorta (Angelucci and Mastelli-Coriandoli, 1958). Evidence has also been reported that i.v. treatment of patients with lipoic acid improves the process of lipid catabolism as well as induces an increase in the serum protein content, suggesting an action of lipoic acid at a common step in the metabolic process of degradation and synthesis of lipids and proteins (Bustamante et al., 1998). Lipoic acid was associated with marked and statistically significant decreases in fibrinogen, factor VII, triglycerides and improved endoneural blood flow in diabetic rats (Ford et al., 2001). These marked effects of lipoic acid on both lipid and hemostatic risk factors could play a protective role in cardiovascular diseases.

To conclude, cyclophosphamide treatment resulted in elevated serum lipids and lipoprotein fractions. Abnormal activities of lipid metabolizing enzymes contributed to these hyperlipidemic changes induced by cyclophosphamide. Lipoic acid was beneficial in restoring the lipidemic status to near normalcy, suggesting that it would be beneficial in hyperlipidemic cardiomyopathy.

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