In vivo studies on the metabolic derangements induced by CP: Role of LA
CHAPTER 2

Heart is the central organ of the circulatory system. Normal cardiac function requires the tight interaction between metabolism, contractile function and gene expression (Depre and Taegtmeyer, 2000). The mammalian heart is an obligate aerobic organ. In the adult heart, the energy is obtained primarily from fatty acid β-oxidation, which is the main source of reducing equivalents and acetyl-CoA for oxidative phosphorylation. However, the other metabolic pathways such as glycolysis, ketone body oxidation also contribute to the generation of ATP (Carvajal and Moreno-Sanchez, 2003). The creatine kinase system is involved in energy transfer from mitochondria to myofibrils.

Mitochondria are vital for the proper function of the adult myocardium to fulfill its function as a circulatory pump and to maintain ion homeostasis. Cardiac myocytes are the cells with the highest volume density of mitochondria in the body due to the extraordinary demand for continuous synthesis of ATP by oxidative metabolism (Goffart et al., 2004). ATP produced by means of oxidative phosphorylation is essentially used for contractile force production. Recent interest focusing on mitochondrial dysfunction reflects the growing consensus, that errors in metabolism often accompany and modify cardiomyocyte dysfunction (Shoffner and Wallace, 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. ApoB-containing lipoproteins appear to serve as an effective means of secreting substantial amounts of triglycerides from cardiomyocytes (Yang and Cheng, 2005). Oxygen plays a vital role in energy metabolism while ROS produced during these reactions can be detrimental to cardiac myocytes.

2.1 REDOX DISEQUILIBRIUM IN THE FAILING HEART

Heart is particularly vulnerable to free radical damage due to low levels of antioxidants that provide cellular defence against ROS (Olson et al., 1981). Redox disequilibrium due to altered production and/or spatiotemporal distribution of reactive oxygen and nitrogen species creates oxidative and/or nitrosative stresses in the failing heart and vascular tree, which contribute to the abnormal cardiac and vascular phenotypes that characterize the failing cardiovascular system and metabolic derangements (Hare and Stamler, 2005).

Modification of proteins by ROS/RNS can cause inactivation of critical enzymes and can induce denaturation that renders proteins nonfunctional. General membrane damage secondary to ROS mediated lipid peroxidation can result in altered ion channel flux and membrane ion pump function in heart muscle (Giordano, 2005). In the setting of acute myocardial infarction (MI), ROS are purported to play a significant role in tissue necrosis and reperfusion injury (Yoshida et al., 2000).

2.2 CYCLOPHOSPHAMIDE INDUCED CARDIOTOXICITY

Acute cardiotoxicity such as cardiac decompensation and cardiomyopathy has been associated with high dose CP therapy (Schimmel et al., 2004). The pathogenesis of acute cardiotoxicity may be attributed to an
increase in free oxygen radicals in oxazaphosphorine induced cardiotoxicity. This increase would be mediated by elevated intracellular levels of the actual cytotoxic metabolite phosphoramidate mustard (Lipp, 1999). CP metabolism may result in free radical generation both in vivo and in vitro (Kanekal and Kehrer, 1994; Sulkowska and Sulkowski, 1997).

CP administration results in cardiac membrane damage resulting in altered permeability (Gardner et al., 1993). Marked elevation of serum cardiac isoenzymes mimicked myocardial infarction on administration of CP (Dow et al., 1993). Decrease in mitochondrial respiratory function and increase in norepinephrine and acetylcholine linked with the genesis of myocardial damage was observed by Hanaki et al. (1990). Hypercholesterolemia, hypertriglyceridemia and impaired secretion of heart lipoprotein lipase have been reported in CP treated rabbits (Loudet et al., 1984). CP cardiotoxicity was associated with lysosomal membrane damage and loss of lysosomal integrity (Sudharsan et al., 2006a). Cardiotoxicity of CP may be controlled by pharmacological interventions that reduce oxidative stress.

2.3 CARDIOPROTECTION BY LA

LA is an ideal antioxidant that directly quenches free radicals, inhibits reactive oxygen-generators and regenerates other antioxidants (Packer et al., 1995). Moreover, after supplementation with LA for 5 weeks, the highest concentration of free LA was found to be in the heart, amongst the various other tissues (Bustamante et al., 1998). A dihydrolipoic acid-dependent activation of the oligomycin-sensitive mitochondrial SH-groups in rat heart mitochondria and mitoplasts was observed, concomitantly, with an activation of ATP-synthase (Zimmer et al., 1991). Besides the antioxidant role, the
marked effects of LA 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 in vivo model has been designed to evaluate the cardiac metabolic changes in CP administered rats. Further, the impact of LA on myocardial damage was evaluated.

2.4 MATERIAL AND METHODS

2.4.1 DRUGS AND CHEMICALS

Cyclophosphamide (Endoxan®) was purchased from German Remedies Limited, Goa, India. DL-α-Lipoic acid, 1,1,3,3 tetaethoxypropane, pyrogallol and bovine serum albumin were procured from Sigma Chemicals, St Louis, MO, USA. Adenosine triphosphate and nicotinamide adenine dinucleotide were purchased from Sisco Research Laboratories Private Limited, Bombay. All other chemicals and solvents used were of highest purity and analytical grade.

2.4.2 EXPERIMENTAL ANIMALS

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 hr light:dark cycle. Animal experiments were
conducted according to the guidelines of institutional animal ethical committee.

2.4.2.1 Study Design

Rats were divided into four groups, each consisting of six animals.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
</tr>
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<tbody>
<tr>
<td>I</td>
<td>Animals served as vehicle treated controls</td>
</tr>
<tr>
<td>II</td>
<td>Animals were injected intraperitoneally with a single dose of CP (200 mg/kg body weight, i.p.) dissolved in saline, on the first day of the experimental period</td>
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<tr>
<td>III</td>
<td>Animals received LA (25 mg/kg body weight) by oral gavage dissolved in saline at alkaline pH (7.8) daily for 10 days</td>
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<tr>
<td>IV</td>
<td>Animals were administered CP as in Group II, immediately followed by administration of LA daily for 10 days</td>
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After the experimental period of 10 days, rats of all the groups were anesthetized and decapitated. Heart was immediately excised and rinsed in ice-cold physiological saline. A 10% homogenate of the heart was prepared in 0.01 M Tris-HCl buffer (pH 7.4) and aliquots of this homogenate were used for the following assays. A section of the heart was kept aside for histological studies. Blood was collected for the analysis of biochemical parameters. The animals were placed in metabolic cages to obtain 24-h urine samples prior to death by decapitation.
2.4.3 ESTIMATION OF PROTEIN

Protein content in serum, heart tissue homogenate and mitochondrial fraction was estimated by the method of Lowry et al. (1951). The aromatic amino acids, tyrosine and tryptophan, present in proteins react with Folin-Ciocalteau reagent containing phosphomolybdic acid and tungstate to produce a blue coloured complex, which absorbs maximally at 620 nm. 0.1 ml of the serum or tissue homogenate was made upto 1 ml with water. To this 4.5 ml of alkaline copper reagent (2% Na₂CO₃ in 0.1 N NaOH was mixed with 0.5% CuSO₄ in 1% sodium potassium tartrate in the ratio of 50:1) was added, mixed and allowed to stand at room temperature for 20 min. Later 0.5 ml of Folin’s phenol reagent was added and shaken well. The blank and standard bovine serum albumin was treated in a similar manner. The blue complex formed was measured at 640 nm after 15 min against the blank.

2.4.4 ENZYMIC MARKERS OF CELLULAR INTEGRITY

2.4.4.1 Lactate Dehydrogenase (E.C.1.1.1.27, LDH)

The method adopted for the estimation of LDH was that of King (1965a). The method was based on the ability of LDH to convert lactate to pyruvate with the help of the coenzyme nicotinamide adenine dinucleotide (NAD⁺). The pyruvate formed was made to react with 2,4-dinitrophenyl hydrazine (DNPH) in hydrochloric acid. The hydrazone formed turns into an orange coloured complex in alkaline medium, which was measured at 420 nm. The activity of LDH was expressed as μmoles of pyruvate liberated/ min/mg protein.
2.4.4.2 **Alanine Transaminase** (E.C.2.6.1.2, ALT)

It was assayed by the method of King (1965b). The method was based on the ability of ALT to form glutamate and pyruvate from the substrate containing alanine and 2-oxoglutaric acid. The pyruvate formed is made to react with DNPH reagent. A set of standard pyruvate was also treated in the similar manner. The colour developed was read at 540 nm after the addition of NaOH. The enzyme activity was expressed as μmoles of pyruvate liberated/min/mg protein.

2.4.4.3 **Aspartate Transaminase** (E.C. 6.1.1, AST)

It was assayed by the method of King (1965b). The method was based on the ability of AST to form glutamate and oxaloacetate from the substrate containing aspartic acid and 2-oxoglutaric acid. The oxaloacetate formed is made to react with DNPH reagent. A set of standard pyruvate was also treated in the similar manner. The colour developed was read at 540 nm after the addition of NaOH. The enzyme activity was expressed as μmoles of pyruvate liberated/min/mg protein.

2.4.4.4 **Creatine phosphokinase** (E.C. 2.7.2.3, CPK)

CPK was assayed in the serum and heart tissue by the method of Okinaka *et al.* (1964). The enzyme catalyses the reversible transphosphorylation reaction between creatine and ATP forming creatine phosphate and ADP. Enzyme activity was expressed as μmoles of phosphorus liberated/min/mg protein.
2.4.5 BIOCHEMICAL ESTIMATIONS

Plasma was treated with 10% sodium tungstate and 2/3 N $\text{H}_2\text{SO}_4$ to precipitate the proteins and centrifuged. Suitable aliquots of the processed plasma and 24 h urine samples were used for the estimation of urea, uric acid and creatinine.

2.4.5.1 Estimation of urea

Urea was estimated by the method of Natelson et al. (1971) in which urea forms a coloured complex with diacetyl monoxime in acidic medium on heating. The colour developed was read at 480 nm. Units were expressed as mg/24 h in urine and mg/dl in serum.

2.4.5.2 Estimation of uric acid

Uric acid was estimated from the absorbance of the blue coloured complex formed with phosphotungstic acid in the presence of sodium carbonate at 650 nm according to the method of Caraway (1965). Units were expressed as mg/24 h in urine and mg/dl in serum.

2.4.5.3 Estimation of creatinine

Creatinine was estimated by the method of Owen et al. (1954) in which creatinine forms a yellow coloured complex with picric acid and sodium hydroxide. The colour developed was read at 540 nm. Units were expressed as mg/24 h in urine and mg/dl in serum.
2.4.6 EVALUATION OF OXIDATIVE STRESS

2.4.6.1 Assay of Lipid Peroxidation (LPO)

LPO was determined by the method of Hogberg et al. (1974). 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. Thiobarbituric acid reactive substances include lipid hydroperoxides and aldehydes in tissue, which are assessed in terms of MDA equivalents.

The reaction mixture for basal LPO consisted of 0.15 M Tris-HCl buffer (pH 7.4), 10 mM KH₂PO₄ and 0.2 ml of tissue extract in a total volume of 2 ml. The tubes were incubated at 37°C for 20 min with constant shaking. The reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid (TCA). The tubes were heated in a boiling water bath for 20 min. The tubes were centrifuged and the colour developed was measured at 532 nm. The ferrous sulphate and ascorbate induced LPO system contained 10 mM ferrous sulphate and 0.2 mM ascorbate as inducers (Devasagayam, 1986). The MDA content of the samples was expressed as nmoles of MDA formed/mg/protein.

2.4.6.2 Estimation of Carbonyl content

The protein carbonyl content in heart tissue was analyzed by DNPH method as described by Levine et al. (1990). Briefly, 1 ml of tissue supernatant containing 0.5 mg protein was added to 4.0 ml of DNPH in 2.5 M HCl. 2.5 M HCl served as blank. Samples were incubated at the room temperature for 1 h. Then, protein was precipitated by adding 5 ml of 20% TCA and washed three times with 4 ml of ethanol:ethyl acetate (1:1).
Precipitated protein was redissolved in 2.0 ml of 6 M guanidine HCl and insoluble substance removed by centrifugation. Carbonyl content was calculated from the maximum absorbance at 366 nm. The results were expressed as nmols of carbonyl/mg protein.

2.4.6.3 Estimation of 8-hydroxydeoxyguanosine (8-OHdG) by High Performance Liquid Chromatography

8-OHdG was estimated by the method of Ito et al. (1993). The DNA of heart tissue was isolated by the manufacturer's instructions provided with the DNA-zol reagent kit. DNA was dissolved in 20 mM acetate buffer (pH 5.0) and digested to deoxynucleosides by incubation first with 8 units of nuclease Pi at 37°C for 30 minutes and then with 1.3 units of calf intestinal alkaline phosphatase at 37°C for 1 h in 0.1 M Tris-HCl buffer (pH 7.5). The resulting deoxynucleosides mixture was injected into a C_{18} Beckman Ultrasphere column of HPLC apparatus equipped with a UV detector and electrochemical detector. 10% aqueous methanol containing 12.5 mM citric acid, 25 mM sodium acetate, 30 mM NaOH and 10 mM acetic acid; flow rate 1 ml/min. Standard 8-OHdG was used to quantify the levels of 8-OHdG levels in the DNA samples.

2.4.6.4 Assay of Enzymic Antioxidants

2.4.6.4.1 Superoxide Dismutase (E.C.1.15.1.1, SOD)

The enzyme was assayed according to the method of Marklund and Marklund (1974). The degree of inhibition of auto-oxidation of pyrogallol, in an alkaline pH by SOD was used as a measure of the enzyme activity.
To the tissue aliquot, 0.25 ml of ethanol and 0.15 ml of chloroform were added. After 15 min of shaking, the suspension was centrifuged and the supernatant obtained, constituted the enzyme extract. The reaction mixture for auto-oxidation consisted of 2 ml of 0.1 mM Tris-HCl buffer (pH 8.2), 0.5 ml of 2 mM pyrogallol in 0.05 M Tris-HCl buffer (pH 7.4) and 1.5 ml of water. Initially the rate of auto-oxidation of pyrogallol was noted at an interval of 1 min to 3 min. The assay mixture for the enzyme contained 2 ml of the buffer, 0.5 ml of pyrogallol, aliquots of the enzyme preparation and water to give a final volume of 4 ml. The rate of inhibition of pyrogallol auto-oxidation after the addition of the enzyme was noted. The enzyme activity is defined as the enzyme required to give 50% inhibition of pyrogallol auto-oxidation/min (units/mg protein).

2.4.6.4.2 Catalase (E.C.1.11.1.6, CAT)

The activity of CAT was assayed by the method of Sinha (1972). The method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of H₂O₂ with the formation of perchloric acid as an unstable intermediate. The chromic acetate was measured at 610 nm. The assay mixture contained 0.5 ml of 0.2 mM H₂O₂, 1 ml of 10 mM sodium phosphate buffer (pH 7.0) and 0.4 ml water. 0.2 ml of the tissue aliquot was added to initiate the reaction. 2 ml of the dichromate/acetic acid reagent (5% solution of potassium dichromate in water with glacial acetic acid in the ratio of 1:3, diluted 1:5 with water) was added after 30 and 60 sec of incubation. To the control tube, the tissue aliquot was added after the addition of the acid reagent. The tubes were then heated for 10
min and colour developed was read at 610 nm. The activity of CAT was expressed as μmoles of H₂O₂ consumed/min/mg protein.

2.4.6.4.3 Glutathione peroxidase (E.C.1.11.1.9, GPx)

The activity of GPx was determined by the modified method of Rotruck et al. (1973). The remaining glutathione after the enzyme catalysed reaction, was complexed with 5,5’dithiobis-(2-nitrobenzoic acid) (DTNB) that absorbs maximally at 412 nm. 0.4 ml of 0.4 M sodium phosphate buffer (pH 7.0), 0.1 ml of 10 mM sodium azide, 0.2 ml of 4 mM GSH, 0.1 ml of 2.5 mM H₂O₂, tissue aliquot and water were taken into a final incubation volume of 2 ml. The tubes were incubated at 37°C for 3 min and the reaction was terminated by the addition of 0.5 ml of 10% TCA. To determine the remaining reduced glutathione (GSH) content, the supernatant was removed by centrifugation and 3 ml of 0.3 M disodium hydrogen phosphate solution and 1 ml of the 0.04% DTNB in 1% sodium citrate was added to 1 ml of supernatant. The colour developed was read at 412 nm. Suitable aliquots of the standard (GSH) were taken and treated in the same manner. The enzyme activity was expressed as μg of GSH consumed/min/mg protein.

2.4.6.5 Estimation of Non-Enzymic Antioxidants

2.4.6.5.1 Total Reduced Glutathione (GSH)

GSH was determined by the method of Moron et al. (1979). This method is based on the reaction of GSH with DTNB to give a compound that absorbs at 412 nm. Tissue homogenates were precipitated with 5% TCA. To an aliquot of the supernatant, 2 ml of 0.6 mM DTNB in 0.2 M sodium phosphate buffer (pH 8.0) and 0.8 ml of 0.2 M sodium phosphate buffer
(pH 8.0) were added to make a final volume of 3 ml. The absorbance was read at 412 nm against a reagent blank. A series of standards treated in a similar manner were also run. The amount of GSH was expressed as µg/mg protein.

2.4.6.5.2 Ascorbic Acid or Vitamin C

Ascorbic acid was estimated by the method of Omaye et al. (1979) using dinitrophenylhydrazine-thiourea-copper sulphate reagent (DNPH-Thiourea-CuSO₄ reagent). Aliquots of homogenate was precipitated with 5% ice-cold TCA and centrifuged for 20 min at 3500 x g. 1 ml of the supernatant was mixed with 0.2 ml of DNPH-Thiourea-CuSO₄ reagent (3 g DNPH, 0.4 g thiourea and 0.05 g CuSO₄ were dissolved in 9N H₂SO₄ and made upto 100 ml with the same) and incubated for 3 h at 37°C. Then 1.5 ml of ice cold 65% H₂SO₄ was added, mixed well and the solutions were allowed to stand at room temperature for an additional 30 min. Absorbance was measured at 520 nm. The values were expressed as µg of ascorbate/mg protein.

2.4.6.5.3 α-Tocopherol or Vitamin E

α-Tocopherol was determined by the method of Desai (1984). The values were expressed as µg/mg tissue.

2.4.6.6 Estimation of Thiols

2.4.6.6.1 Total sulfhydryl groups (TSH)

The level of TSH was estimated by the method of Sedlak and Lindsay (1968). In this method, DTNB was reacted with the tissue homogenate,
wherein DTNB is reduced by the thiol (-SH) group to form 1 mole of 2-nitro-5-mercaptobenzoic acid per mole -SH. The absorbance of the supernatant was read at 412 nm. The concentration of TSH was expressed as \( \mu g/mg \) tissue using the calibration curve obtained with GSH as standard.

### 2.4.6.6.2 Non-protein sulphhydril groups (NPSH)

NPSH were estimated by the method of Sedlak and Lindsay (1968). The tissue homogenate was mixed with 10% TCA to precipitate the proteins and centrifuged. DTNB was reacted with the supernatant and the colour intensity was measured at 412 nm. The concentration of NPSH was expressed as \( \mu g/mg \) tissue using the calibration curve obtained with GSH as standard.

### 2.4.6.7 Assay of Glutathione Metabolising Enzymes

#### 2.4.6.7.1 Glutathione S-transferase (E.C.2.5.1.18, GST)

This enzyme was assayed by the method of Habig et al. (1974). GST catalyses the reaction of 1-chloro-2,4-dinitrobenzene (CDNB) with the sulphhydril group of glutathione to form the conjugate, CDNB-glutathione that absorbs light at 340 nm. Briefly, the reaction mixture contained 0.1 M potassium phosphate buffer (pH 6.5), 1 mM CDNB and 1 mM GSH. The reaction was started by the addition of enzyme and the increase in optical density at 340 nm was measured against that of the blank. The activity of the enzyme can therefore be estimated by measuring the increase in the optical density at 340 nm. GST activity was expressed as nmoles of CDNB-GSH conjugate formed/min/mg protein.
2.4.7.2 Glutathione Reductase (E.C.1.6.4.2, GR)

The enzyme was assayed by the method of Staal et al. (1969). The decrease in optical density at 340 nm due to oxidation of NADPH with oxidized glutathione (GSSG) as an acceptor was followed. GR activity was determined in 0.2 M sodium phosphate buffer (pH 6.8) and 5 mM EDTA in a final volume of 2.5 ml containing 1mM GSSG, 120 μM NADPH and enzyme. The decrease in OD at 340 nm at 37°C was measured against the blank for 3 min at 1 min intervals. Activity of GR was expressed as nmoles of NADPH oxidized/min/mg protein.

2.4.7 MEMBRANE ATPases

2.4.7.1 Na⁺, K⁺-Adenosine Triphosphatase (E.C. 3.6.1.3, Na⁺, K⁺-ATPase)

Na⁺,K⁺-ATPase activity was estimated by the method of Bonting (1970). The phosphorus content was estimated by the method of Fiske and Subbarow (1925). The enzyme activity was expressed as μmoles of Pi liberated/min/mg protein.

2.4.7.2 Ca²⁺-Adenosine Triphosphatase (E.C. 3.6.1.3, Ca²⁺-ATPase)

The activity of the enzyme was estimated according to the method of Hjerten and Pan (1983). The amount of Pi liberated was estimated by the method of Fiske and Subbarow (1925). The enzyme activity was expressed as μmoles of Pi liberated/min/mg protein.

2.4.7.3 Mg²⁺-Adenosine Triphosphatase (E.C. 3.6.1.3, Mg²⁺-ATPase)

The activity of the enzyme was estimated according to the method of Ohinishi et al. (1982). The liberated Pi was estimated by the method of Fiske
and Subbarow (1925). The enzyme activity was expressed as μmoles of Pi liberated/min/mg protein.

2.4.8 ASSAY OF NITRITE AND NITRATE

The concentration of Nitric Oxide (NO) in a system was measured by the determination of total nitrate and nitrite concentrations in the sample (R&D Systems, Minneapolis, USA). This assay determines total nitric oxide, based on the enzymatic conversion of nitrate to nitrite by nitrate reductase. The reaction is followed by a colorimetric detection of nitrite as an azo dye product of the Griess reaction. The Griess reaction is based on the two-step diazotization reaction in which acidified nitrite produces a nitrosating agent, which reacts with sulphanilic acid to produce the diazonium ion. This ion is then coupled to N-(1-naphthyl)ethylenediamine to form the chromophoric azo-derivative which absorbs light at 540 - 570 nm.

2.4.9 EXPRESSION OF iNOS BY REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR)

2.4.9.1 Extraction of total RNA

Total RNA from heart tissue was isolated according to the manufacturer’s instruction (Trizol, One step RNA isolation kit, Medox Biotech Pvt Ltd.). Briefly, immediately after the sacrifice, 50 mg of minced heart tissue was homogenized in 1 ml of one step RNA reagent containing 0.25 g glycogen. The homogenate was cleared off by centrifugation for 10 min at 12,000 x g and the resulting supernatant was transferred into a 1.5 ml microcentrifuge tube followed by the addition of 200 μl of chloroform to the lysate and shaken well for 15-25 sec and incubated at 15-30°C for
3-5 min and then centrifuged for 15 min at 12,000 x g. Then, the aqueous layer was transferred into 1.5 ml microcentrifuge tube and added 500 µl of ice cold isopropanol. The tube was kept for precipitation of RNA at -20°C for 1-2 h and centrifuged for 10-15 min at 12,000 x g. Then, the RNA pellet was washed twice by adding 1 ml of 75% ethanol and centrifuged at 7,500 x g for 5-10 min. The pellet was allowed to air dry, then the RNA pellet was dissolved in 50-100 µl RNase free water. The RNA was stored at -80°C. All the reactions were carried out at 4°C unless otherwise stated. Subsequently, the purity and yield of RNA was done by measuring the absorbance of RNA solution at 260 nm and 280 nm (Absorbance ratio of 260/280 ranges from 1.6-1.8 was taken for further reaction).

2.4.9.2 Reverse Transcription-Polymerase Chain Reaction (RT-PCR) for iNOS mRNA expression

RT-PCR for iNOS-mRNA expression was done according to manufacturing guidelines (Qiagen One Step RT-PCR mix). Briefly the reaction mixture contained 10 µl of 5x Qiagen One step RT-PCR Buffer containing final concentration of 2.5 mM MgCl₂, 2 µl of dNTP Mix (0.4 mM of each dNTP as final concentration), 5 µl of each sense and antisense primers of iNOS, 5 µl of sense and antisense primers of housekeeping RPL-19 (each of 0.6 µM final concentration), 0.75 µg of template RNA, 2 µl of Qiagen One step RT-PCR enzyme mix and made upto 50 µl with RNase free water. During reverse transcription, HotstarTaq DNA polymerase in the enzyme mix is inactive while after reverse transcription, reactions were heated to 94°C for 15 min to activate DNA polymerase and to simultaneously inactivate the reverse transcriptase. Reverse transcription was carried out at 50°C for
60 min. After this reaction, initial PCR activation step was carried out at 94°C for 15 min to inactivate reverse transcriptase and denature cDNA template. This was followed by the 3 step cycling with denaturation at 94°C for 15 sec, primer annealing at 60°C for 30 sec and extension at 72°C for 30 sec. A total of 39 cycles were performed. Final extension was carried out at 72°C for 5 min. Then the reaction was terminated by holding the temperature at 4°C. The RT-PCR products were electrophoresed by using 2% agarose gel and quantified densitometrically using housekeeping as internal control.

2.4.9.3 List of primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Product size (bp)</th>
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<tbody>
<tr>
<td>iNOS</td>
<td>Sense - 5’ - TCT GTG CCT TTG CTC ATG AC – 3’</td>
<td>305</td>
</tr>
<tr>
<td></td>
<td>Antisense - 5’ - CAT GGT GAA CAC GTT CTT GG – 3’</td>
<td></td>
</tr>
<tr>
<td>RPL-19</td>
<td>Sense- 5’ – CTG AAG GTC AAA GGG AAT GTG – 3’</td>
<td>194</td>
</tr>
<tr>
<td></td>
<td>Antisense- 5’ – GGA CAG AGT CTT GAT GAT CTC – 3’</td>
<td></td>
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2.4.10 ESTIMATION OF MINERALS

The serum and cardiac tissue were digested according to the method of Ballentine and Burford (1957). Serum and a weighed amount of the cardiac tissues were taken in a Pyrex kjeldahl flask. To this 1 ml of concentrated HNO₃ was added, followed by 1 ml of perchloric acid. This content was digested over a sand bath until the solution became pale yellow in colour. When the colour of the digest was brown, additional HNO₃ and perchloric acid were added and the oxidation was repeated. The digest was made up to a
known volume with deionized water. Aliquots of this were used to estimate the inorganic constituents. Calcium and magnesium were estimated by sequential plasma emission spectrophotometry (ARL Model 3410 ICP spectrometer system).

The basis for emission spectrometry is that atoms or ions in an energized state spontaneously revert to a lower energy state and in doing so emit a photon of energy. For quantitative emission spectrometry, it is assumed that the emitted energy is proportional to the concentration of atoms (or) ions to be measured.

2.4.11 ASSAY OF LYPOSOMAL ENZYMES

2.4.11.1 Acid phosphatase (E.C. 3.1.3.2, ACP)

The enzyme was assayed by the method of King (1965c) using disodium phenyl phosphate (DSPP) as the substrate. The incubation mixture contained 1.5 ml of 0.1 M citrate buffer (pH 4.8), 1.5 ml of 10 mM DSPP and enzyme aliquot. After incubation at 37°C for 30 min, the reaction was arrested by the addition of 1 ml of 10% TCA. To the supernatant, 1 ml of 15% sodium carbonate and 0.5 ml of Folin's Ciocalteau were added and incubated for 10 min. The colour developed was read at 640 nm. The enzyme activity was expressed as μmoles of phenol liberated/h/mg protein.

2.4.11.2 β-N-Acetylglucosaminidase (E.C. 3.2.1.30, NAG)

NAG activity was assessed by the method of Maruhn (1976) using 4-nitrophenyl-N-acetyl glucosaminide as the substrate. The enzyme activity was expressed as μmoles of p-nitrophenol formed/h/mg protein.
2.4.11.3 β-Glucuronidase (E.C. 3.2.1.31, β-Glu)

β-Glu was estimated by the method of Kawai and Anno (1971). The substrate for the enzyme reaction was p-nitrophenyl β-D-glucuronide and the enzyme activity was assessed in terms of µmoles of p-nitrophenol liberated/h/mg protein.

2.4.11.4 β-Galactosidase (E.C. 3.2.1.23, β-Gal)

β-Gal was estimated by the method of Kawai and Anno (1971). The colour developed was read at 400 nm against a reagent blank. Standards were also run simultaneously. The activity was expressed as µmoles of p-nitrophenol liberated/h/mg protein.

2.4.11.5 Cathepsin-D (E.C. 3.4.23.5, Cat-D)

Cat-D was estimated by the method of Etherington (1972). The incubation mixture contained the tissue homogenate or plasma and buffered substrate (1.5% hemoglobin in sodium acetate buffer). The colour developed was read at 640 nm and the enzyme activity was expressed as µmoles of tyrosine liberated/h/mg protein.

2.4.12 HYDROXYPROLINE LEVEL IN THE CARDIAC TISSUE

Hydroxyproline levels in the heart were determined by the method of Woessner (1961). Cardiac tissue was homogenized in 2 ml distilled water and incubated with 125 µl of 50% TCA on ice for 20 min. Samples were centrifuged and the pellet was mixed with 1 ml of 12 N HCl and baked at 110°C for 14-18 h until samples were charred and dry. The samples were
resuspended in 2 ml of deionized water by incubating for 72 h at room temperature applying intermittent vortexing. Serial dilutions of trans-4-hydroxy-L-proline standard were prepared starting at 0.5 mg/ml. 200 ml of vortexed sample (or standard) was added to 500 ml of 1.4% chloramine T in 0.5 M sodium acetate/10% isopropanol and incubated for 20 min at room temperature. Next, 500 ml of Ehrlich's solution (1.0 M p-dimethylaminobenzaldehyde in 70% isopropanol/30% perchloric acid was added, mixed and incubated at 65°C for 15 min. After samples returned to room temperature, the optical density of each sample and standard was measured at 550 nm and the concentration of heart OH-P was calculated from the OH-P standard curve. The OH-P was calculated and the units were expressed in mg/g dry tissue.

2.4.13 ISOLATION OF HEART MITOCHONDRIA

Heart mitochondria were isolated by the method of Takasawa et al. (1993). The heart tissue was put into ice cold 50 mM Tris-HCl (pH 7.4) containing 0.25 M sucrose and homogenized. The homogenates were centrifuged at 700 x g for 20 minutes, and then the supernatants obtained were centrifuged at 9000 x g for 15 minutes. Then the pellets were washed with 10 mM Tris-HCl (pH7.8) containing 0.25 M sucrose and finally resuspended in the same buffer.

2.4.13.1 TCA cycle enzymes

2.4.13.1.1 Isocitrte dehydrogenase (E.C. 1.1.1.42, ICDH)

The activity of ICDH was estimated by the method of Bernt and Bergmeyer (1974). The amount of isocitrate oxidized was measured by the
increase in extinction due to the formation of NADPH. The enzyme activity was expressed as nmoles of NADPH formed/min/mg protein.

2.4.13.1.2 Succinate dehydrogenase (E.C. 1.3.99.1, SDH)

SDH was assayed by the method of Slater and Borner (1952), in which the rate of reduction of potassium ferricyanide was measured by following the decrease in optical density at 400 nm, in the presence of sufficient potassium cyanide to inhibit cytochrome c oxidase. A 1 cm cell contained sodium succinate, potassium cyanide and potassium ferricyanide in a total volume of 2.8 ml. The reference cell contained water only. At zero time, 0.2 ml of diluted mitochondrial fraction was added to both the cells and optical density at 400 nm was followed as a function of time. The decrease in optical density was recorded as a function of time. The enzyme activity was expressed as nmoles of succinate oxidised/min/mg protein.

2.4.13.1.3 Malate dehydrogenase (E.C. 1.1.1.37, MDH)

The activity of MDH was determined by the method of Mehler et al. (1948). Oxaloacetate was used as the substrate and the enzyme activity was determined by measuring the rate of oxidation of NADH in the presence of enzyme and excess of substrate. The reaction mixture contained 75 μM sodium phosphate buffer, 0.15 μM NADH, 0.76 μM oxaloacetate and 0.2 ml of diluted mitochondrial fraction in a total volume of 3 ml. The reaction was started by the addition of NADH and the change in optical density at 340 nm was measured for 2 min. The enzyme activity was expressed as nmoles of NADH oxidised/min/mg protein.
2.4.13.2 **Enzyme complexes of Electron Transport Chain**

2.4.13.2.1 **NADH dehydrogenase or Complex I: NADH-Ubiquinone oxidoreductase (E.C. 1.6.99.3)**

The activity of Complex I was determined by the method of Minakami *et al.* (1962) in which the rate of NADH oxidation was monitored at 340 nm. The enzyme activity was expressed as µmoles of NADH oxidised/min/mg protein.

2.4.13.2.2 **Succinic-coenzyme Q or complex II: Succinate-Ubiquinone oxidoreductase (E.C. 1.3.5.1)**

The activity of complex II was assayed by the method of Ziegler and Doeg (1962). The reaction was initiated with CoQ and the rate of reduction of 2,6-dichlorophenolindophenol (DCIP) was recorded at 600 nm. The enzyme activity was expressed as µmoles of DCIP reduced/min/mg protein.

2.4.13.2.3 **Coenzyme Q-cytochrome reductase or Complex III: Ubiquinol ferricytochrome c oxidoreductase (E.C. 1.10.2.2)**

The activity of Complex III was determined by the method of Green and Burkhard (1961) by measuring the rate of reduction of ferricytochrome by the reduced form of coenzyme Q at 550 nm. The enzyme activity was expressed as µmoles of cytochrome c reduced/min/mg protein.

2.4.13.2.4 **Cytochrome c oxidase or Complex IV: Ferrocytochrome c–Oxygen oxidoreductase (E.C. 1.9.3.1)**

The specific activity of Complex IV was assayed by the method of Wharton and Tzagaloff (1967). The rate of oxidation of ferrocytochrome was
measured as the rate of decrease in absorbance at 550 nm. The enzyme activity was expressed as μmoles of cytochrome c oxidised/min/mg protein.

2.4.14 LIPIDS PROFILE

2.4.14.1 Preparation of tissue lipid extract

A weighed amount of tissue (500 mg) was homogenized with 7 ml of methanol in a Potter-Elvehjem homogeniser and filtered through a Whatman No.1 filter paper into a conical flask. The residue after filtration was scraped and homogenized in 14 ml of chloroform. The residue was once again scraped from the filter paper and ground with 10 ml of chloroform-methanol mixture (2:1 v/v) and the resulting filtrate was evaporated to dryness.

The lipids were purified by the Folch Wash procedure (Folch et al., 1957). The dried lipid residue after evaporation was dissolved in 5 ml of chloroform-methanol mixture. The redissolved lipid extract was mixed with 1 ml of 0.1 N KCl and the contents were shaken well. The upper aqueous phase containing gangliosides and other water-soluble compounds were separated. The lower chloroform phase, containing neutral and phospholipids was again washed thrice with 2 ml of Folch’s reagent (0.1N KCl: Methanol:Chloroform mixed in the ratio 10:10:1) and the upper aqueous phase was aspirated. The lower chloroform phase was made up to a known volume and aliquots were taken for the analysis of lipids.

2.4.14.2 Cholesterol

Cholesterol was estimated by the method of Parekh and Jung (1970). An aliquot of the serum sample or the dried tissue lipid extract was reacted
with ferric chloride-uranyl acetate reagent followed by sulphuric acid-ferrous sulphate reagent. The colour developed was read at 540 nm. The total cholesterol concentration was expressed as mg/dl serum and as mg/g wet tissue.

2.4.14.3 Free cholesterol (FC)

FC was precipitated as its digitonide according to the method of Sperry and Webb (1950) and cholesterol in the precipitate was estimated by the method of Parekh and Jung (1970). The ester cholesterol was arrived at from the difference between the total and free cholesterol values. The levels of free and ester cholesterol concentrations were expressed as mg/dl serum and as mg/g wet tissue.

2.4.14.4 Triglycerides (TG)

TG were estimated by the method of Rice (1970). The level of TGs in serum was expressed as mg/dl and in tissue as mg/g wet tissue.

2.4.14.5 Phospholipids (PL)

PL were estimated by the method of Rouser et al. (1970) after digesting the lipid extract with perchloric acid (Bartlett, 1959). The colour developed was read at 700 nm. PL content was expressed as mg/dl serum and as mg/g wet tissue.
2.4.14.6 Free fatty acids (FFA)

FFA were measured by the method of Hron and Menahan (1981). The colour developed with diethyl dithiocarbamate was read at 420 nm. The level of FFA in serum was expressed as mg/dl and in tissue as mg/g wet tissue.

2.4.15 LIPOPROTEINS

The lipoproteins were fractionated by a dual precipitation technique (Wilson and Spiger, 1973). After the fractional precipitation, the lipoprotein cholesterol was estimated (Parekh and Jung, 1970).

About 1 ml of serum was added to 0.18 ml of Heparin-manganese chloride reagent (3.167 g of MnCl₂ was added to 1 ml solution of heparin containing 5000 Units and made upto 8 ml with water). The solution was incubated at 4°C for 30 min. It was then centrifuged at 11,000 x g for 30 min and the supernatant contained HDL.

To 1 ml of serum, 0.15 ml of 10% Sodium dodecyl sulphate (SDS) in 0.15 sodium chloride was added. VLDL flocculated on top. The contents were centrifuged at 10,000 x g for 15 min. The supernatant contained both HDL and LDL. The cholesterol content of each fraction was arrived as follows:

\[
\text{VLDL cholesterol} = \text{Total cholesterol} - \text{supernatant from SDS (LDL and HDL)}
\]

\[
\text{LDL cholesterol} = \text{Supernatant from SDS (LDL and HDL)} - \text{heparin Mn}^{2+} \text{ (HDL)}
\]

Lipoprotein cholesterol was expressed as mg/dl of serum.
2.4.16 LIPID METABOLIZING ENZYMES

2.4.16.1 Lipoprotein lipase (E.C. 3.1.1.3, LPL)

LPL was assayed by the method of Schmidt (1974). The colour developed was read at 430 nm. LPL activity was expressed as μmoles of free fatty acids liberated/h/mg protein.

2.4.16.2 Lecithin:Cholesterol Acyl Transferase (E.C. 2.3.1.43, LCAT)

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 μmoles of cholesterol esterified/h/mg protein.

2.4.16.3 Cholesterol Ester Synthetase (E.C. 2.3.1.26, CES)

CES was assayed by the method of Kothari et al. (1973). The colour developed was read at 540 nm. Enzyme activity was expressed as nmoles of cholesterol esterified/h/mg protein.

2.4.16.4 Cholesterol Ester Hydrolase (E.C. 3.1.1.13, CEH)

CEH activity was estimated by the method of Kothari et al. (1970) with slight modification by Kritchevsky and Kothari (1973). The free cholesterol liberated from cholesterol oleate was precipitated and the cholesterol content estimated. The colour developed was read at 540 nm. The enzyme activity was expressed as nmoles of cholesterol liberated/h/mg protein.
2.4.17 MORPHOLOGICAL STUDIES

2.4.17.1 Histopathologic Studies

Portions of heart tissues were fixed in 10% formalin. The washed tissues were dehydrated in the descending grades of isopropanol and finally cleared in xylene. The tissues were then embedded in molten paraffin wax. Sections were cut at 5 μm thickness, stained with haematoxylin and eosin. The sections were then viewed under light microscope for histopathological changes.

2.4.18 STATISTICAL ANALYSIS

The results are expressed as mean values ± standard deviation (S.D) for six animals in each group. Differences between groups were assessed by ANOVA using the SPSS (Statistical Package for Social Sciences) 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.

2.5 RESULTS AND DISCUSSION

CP, one of the most widely prescribed antineoplastic drugs causes a lethal cardiotoxicity. Administration of intermittent doses of CP has been found to be advantageous in chemotherapy (O'Connel and Bennenbaum, 1974). When the differences in body surface area of rats and the humans are taken into account, CP administered at a dose of 200 mg/kg body weight to rats corresponds to the doses typically administered to humans for cancer
chemotherapy and immunosuppression (Wheeler et al., 1962). Appelbaum et al. (1976) have reported that acute myocarditis occurred after 5-9 days, after high dose chemotherapy comprising of CP.

2.5.1 BODY WEIGHT

Table 2.1 shows the body and organ weight changes in the experimental rats. A significant reduction in body weight \( (P < 0.01) \) was noted in Group II animals, which were treated with the cytotoxic drug CP. The reduction in body weight after CP administration may be due to the cytotoxicity of CP, which causes DNA damage and disruption of cell growth (Fraiser et al., 1991). In the present study increase in heart weight \( (P < 0.05) \) was noted in CP treated group. Hypertrophy and fluid accumulation due to myocardial oedema may be responsible for the increase in heart weight (Appelbaum et al., 1976; Deglin et al., 1977). LA supplementation prevented these abnormal changes in heart and body weight of CP administered rats.

2.5.2 MARKERS OF CELLULAR INTEGRITY

CP administration significantly increased \( (P < 0.001) \) the activities of serum CPK, LDH, AST and ALT (Figure 2.1). These observations are consistent with previous reports (Mathew and Kuttan, 1997; Al-Nasser, 1998). 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 (Mathew et al., 1985). Simultaneously, the activities of marker enzymes CPK, LDH, AST and ALT in cardiac tissue were decreased by 46.15\%, 35.28\%, 44.28\% and 29.9\%

* Data published. Please see Annexure
Table 2.1  Effect of cyclophosphamide and lipoic acid on body and heart weight

<table>
<thead>
<tr>
<th></th>
<th>Initial body weight (g)</th>
<th>Final body weight (g)</th>
<th>Change in body weight (g)</th>
<th>Heart weight (g)</th>
<th>Relative heart weight (g%)</th>
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</thead>
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<tr>
<td></td>
<td>141.50 ± 5.99</td>
<td>143.17 ± 3.31</td>
<td>140.5 ± 6.59</td>
<td>0.51 ± 0.04</td>
<td>0.33 ± 0.03</td>
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<td></td>
<td>143.17 ± 3.31</td>
<td>143.88 ± 3.33 a**</td>
<td>153.33 ± 6.80</td>
<td>0.58 ± 0.05 a*</td>
<td>0.40 ± 0.03 a***</td>
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<td>153.50 ± 6.12 b**</td>
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<td></td>
<td>12.67 ± 1.37</td>
<td>0.72 ± 0.10 a***</td>
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<td></td>
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<td>12.83 ± 1.33</td>
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<td></td>
<td></td>
<td></td>
<td>10.83 ± 1.17 a<em>b</em>**</td>
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</table>

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. The symbols ***, ** and * represent statistical significance at $P < 0.001$, $P < 0.01$ and $P < 0.05$ respectively.
Figure 2.1  Effect of cyclophosphamide and lipoic acid on the activities of cardiac marker enzymes in serum

Results are given as mean ± SD for six rats. Units: CPK μmoles x 10⁻² of phosphorus liberated/min/mg protein, LDH μmoles of pyruvate liberated/min/mg protein, AST and ALT μmoles x 10⁻² of pyruvate liberated/min/mg protein. Comparisons are made between a – Group I and Groups II, III, IV, b – Group II and Group IV. The symbols *** and * represent statistical significance at  P < 0.001 and P < 0.05 respectively.
respectively in Group II when compared to control group (Table 2.2). This may be attributed to the release of the intracellular enzymes due to the disruption of myocardial sarcolemma. Key adaptive changes in a failing heart involve a decrease in enzyme activity of the above proteins involved in energy metabolism (Gwathmey et al., 1999).

CPK is a sulphydryl containing enzyme that is particularly susceptible to oxidative inactivation (Koufen and Stark, 2000). Free radicals produced by phosphoramidemustard, a metabolite of CP may inactivate this enzyme. Mitochondrial and cytosolic creatine kinases are involved in the direct transfer of ATP generated from mitochondria to myofibrils. Decrease in the activity of CPK can contribute to the pathogenesis of heart failure by altering energy fluxes, muscular contraction and calcium homeostasis (De Souza et al., 1999). LDH serves as a marker for membrane integrity. In normal cardiac tissue, isoenzymes LDH1 and LDH2 predominate. Decline in the total LDH activity denoting decreased glycolytic capacity (Sylven et al., 1988) may indicate declined left ventricular systolic function caused by CP (Gottdiener et al., 1981). Moreover in the failing cardiomyopathic heart, there is an anaerobic shift in the LDH isoenzymes (Schultheiss, 1992). AST, another marker for membrane integrity has been reported to be reduced in the necrotic myocardium (Siegel et al., 1984). Decline in the activities of aminotransferases may indirectly affect the formation of high energy phosphates via the intermediates of TCA cycle. Activities of the marker enzymes were restored to near normalcy after LA administration. LA interacts with the membrane lipid bilayer (Kagan et al., 1992), maintains cellular integrity, reduces loss of enzymes thereby improving muscle

* Data published. Please see Annexure
### Table 2.2 Effect of cyclophosphamide and lipoic acid on marker enzymes in heart tissue

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<table>
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</thead>
<tbody>
<tr>
<td>CPK</td>
<td>0.65 ± 0.07</td>
<td>0.35 ± 0.03 a***</td>
<td>0.60 ± 0.06</td>
<td>0.59 ± 0.05 b***</td>
</tr>
<tr>
<td>LDH</td>
<td>22.25 ± 2.23</td>
<td>14.40 ± 1.33 a***</td>
<td>21.67 ± 1.86</td>
<td>20.42 ± 1.63 b***</td>
</tr>
<tr>
<td>AST</td>
<td>5.33 ± 0.56</td>
<td>2.97 ± 0.28 a***</td>
<td>5.12 ± 0.53</td>
<td>5.02 ± 0.56 b***</td>
</tr>
<tr>
<td>ALT</td>
<td>4.38 ± 0.41</td>
<td>3.07 ± 0.29 a***</td>
<td>4.12 ± 0.44</td>
<td>3.90 ± 0.46 b**</td>
</tr>
</tbody>
</table>

Results are given as mean ± S.D for six rats. Units: CPK: μmoles of phosphorus liberated/min/mg protein; LDH: μmoles x 10⁻¹ of pyruvate liberated/min/mg protein; AST and ALT: μmoles x 10⁻² of pyruvate liberated/min/mg protein. Comparisons are made between: a - Group I and Groups II, III, IV; b - Group II and Group IV. The symbols *** and ** represent statistical significance at $P < 0.001$ and $P < 0.01$ respectively.
contractile function and flexibility. This observation is in line with previous reports where LA has been shown to be cardioprotective in ischemia reperfusion injury (Schonheit et al., 1995; Friesleben, 2000).

2.5.3 BIOCHEMICAL VARIABLES

The levels of urea, uric acid and creatinine were increased 1.5 to 2 fold in the serum and urine of CP treated rats (Figures 2.2 and 2.3). Elevated urea, uric acid and creatinine serve as simple yet reliable identifiers of myocardial infarction (Bigger et al., 1978). Hyperuricemia observed in the present study has been reported to be an independent predictor of cardiovascular risk (Ward, 1998). Although the mechanisms by which uric acid may play a pathogenic role in cardiovascular disease is unclear, hyperuricemia is associated with deleterious effects on endothelial dysfunction, oxidative metabolism, platelet adhesiveness, hemorheology and aggregation. (Alderman and Aiyer, 2004). Uric acid produced by xanthine oxidase has antioxidant activity but at high concentrations it acts as a prooxidant. Myocardial infarction is associated with impaired renal function which is reflected in the abnormalities in urine (Gibson et al., 2003). These abnormalities were reverted to near normalcy after LA supplementation.

2.5.4 OXIDATIVE STRESS

Free radicals cause membrane injury by initiating LPO which results in loss of function and integrity of myocardial membranes. 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
Figure 2.2  Effect of cyclophosphamide and lipoic acid on serum urea, uric acid and creatinine levels

Results are given as mean ± S.D for six rats. Units: Urea mg x 10/dl, uric acid and creatinine mg/dl. Comparisons are made between: a - Group I and Groups II, III, IV, b - Group II and Group IV. The symbols ***,** and * represent statistical significance at \( P < 0.001 \), \( P < 0.01 \) and \( P < 0.05 \) respectively.
Figure 2.3  Effect of cyclophosphamide and lipoic acid on urinary urea, uric acid and creatinine levels

Results are given as mean ± S.D for six rats. Units: Urea and creatinine mg/24 h urine, uric acid mg x 10⁻¹/24 h urine. Comparisons are made between: a – Group I and Groups II, III, IV, b – Group II and Group IV. The symbols *** and ** represent statistical significance at $P < 0.001$ and $P < 0.01$ respectively.
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. LA (Group IV) significantly \((P < 0.001)\) prevented the LPO induced by CP. As DHLA scavenges peroxyl radicals that initiate lipid peroxidation, in both membranous and aqueous phases, it effectively prevents the damage of cell membranes by lipid peroxides (Kagan et al., 1992).

Carbonyl content is measured as an index of protein oxidation, where the amino acids are converted to carbonyl derivatives. The heart tissue of CP treated rats showed a 1.51-fold increase in carbonyl content when compared to control rats (Figure 2.5). It is well known that proteins are susceptible to damage by ROS \textit{in vitro} and \textit{in vivo} and oxidative modification of proteins may lead to the structural alteration and functional inactivation of many enzyme proteins (Davies, 1988). Normally, oxidatively modified proteins are degraded more rapidly than native proteins by proteolytic system (Shoal and Brink, 1992), which is proposed as a secondary free radical defense system (Nohl and Hegner, 1978). Increase in carbonyl content may also be due to the decreasing rate of oxidized protein degradation. LA supplementation (Group IV) significantly \((P < 0.001)\) reduced the carbonyl content in heart tissue after CP administration.

8-OHdG is one of several DNA adducts and deletions that accumulate over the life span of an individual (Ames et al., 1989). The heart tissue of CP treated rats showed a significant increase \((P < 0.001)\) in the levels of 8-OHdG when compared to control rats (Figure 2.6). At least three modified bases,
Figure 2.4  Effect of cyclophosphamide and lipoic acid on cardiac lipid peroxidation

Basal  Ascorbate-induced  Ferrous sulphate-induced

<table>
<thead>
<tr>
<th>Group</th>
<th>Basal</th>
<th>Ascorbate-induced</th>
<th>Ferrous sulphate-induced</th>
</tr>
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<tbody>
<tr>
<td>I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>a***</td>
<td>b***</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
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Results are given as mean ± SD for six rats. Comparisons are made between a - Group I and Groups II, III, IV, b - Group II and Group IV. The symbols *** and ** represent statistical significance at $P < 0.001$ and $P < 0.01$ respectively.
Figure 2.5  Effect of cyclophosphamide and lipoic acid on carbonyl content in the heart tissue

Results are given as mean ± SD for six rats. Comparisons are made between a-Group I and Groups II, III, IV, b – Group II and Group IV. The symbol *** represents statistical significance at $P < 0.001$. 
Figure 2.6  Effect of cyclophosphamide and lipoic acid on 8-hydroxydeoxyguanosine levels in heart tissue

Results are given as mean ± SD for six rats. Comparisons are made between: a - Group I and Groups II, III, IV, b - Group II and Group IV. The symbol *** represents statistical significance at $P < 0.001$. 
8-OHdG, 5-hydroxymethyluracil, and thymine glycol, are formed when OH· radical attacks a DNA molecule (Floyd and Schneider, 1990). 8-OHdG appears to be a sensitive and integral marker of oxidative damage to DNA (Cordis et al., 1998). It indicates the generation of free radical species of oxygen, including the hydroxyl free radical, which hydroxylates deoxyguanosine preferentially at C8 (Dizdaroglu, 1991). 8-OHdG adducts accumulate to varying proportions in different tissues, being most prevalent in mitotically fixed, metabolically active tissues such as muscle and brain (Arnheim and Cortopassi, 1992). They are transported through the blood and excreted in the urine (Tagesson et al., 1995). In normal cells, damaged sequences of DNA are rapidly repaired through a process of excision, which is catalysed by exonucleases. If for any reason, DNA is left unreppaired, it may lead to mutagenicity (Floyd and Schneider, 1990). LA supplementation to CP administered rats showed remarkable decrease ($P < 0.001$) in 8-OHdG levels indicating mitigation of oxidative DNA damage by the antioxidant LA. LA could reduce hydroxyl ion induced 8-OHdG by Fenton reagents, chromium (III) (as CrCl3) and hydrogen peroxide (Cr (III)/H2O2) (Lopez-Burillo et al., 2003). The potential of LA in scavenging hydroxyl radicals and chelating transition metals (Packer et al., 1995) could play a key role in reducing the levels of 8-OHdG in cardiac tissue.

The myocardium has a variety of endogenous antioxidants. The major antioxidant enzymes SOD, CAT and GPx act in coordination and provide cellular defense against ROS (Figure 2.7). Statistically significant ($P < 0.001$) decrease in the activities of antioxidant enzymes (SOD, CAT and GPx) was observed in Group II (Table 2.3)\(^\dagger\). Decline in the activities of these enzymes

\(^\dagger\) Data published. Please see Annexure
AT11 (angiotensin II) binds a G-protein-associated receptor, initiating a cascade of events that involves activation of 0^2- production by the NAD(P)H oxidase NOX2. O^2- is converted by SOD into H_2O_2 and 'OH that mediates activation of MAPKs via a tyrosine kinase leading to cardiac hypertrophy or apoptosis. The ROS can also signal through ASK-1 to induce cardiac hypertrophy, apoptosis, or phosphorylate troponin T, an event that reduces myofilament sensitivity and cardiac contractility. NO production by iNOS and eNOS can interact with O^2- to form ONOO which can cause LPO, altering ion channel and ion pump function. Cat and GPx are shown as enzymatic pathways to produce water and oxygen from H_2O_2.

Figure 2.7 Mechanisms by which ROS can alter the structure and function of cardiac muscle. AT11 (angiotensin II) binds a G-protein-associated receptor, initiating a cascade of events that involves activation of 0^2- production by the NAD(P)H oxidase NOX2. O^2- is converted by SOD into H_2O_2 and 'OH that mediates activation of MAPKs via a tyrosine kinase leading to cardiac hypertrophy or apoptosis. The ROS can also signal through ASK-1 to induce cardiac hypertrophy, apoptosis, or phosphorylate troponin T, an event that reduces myofilament sensitivity and cardiac contractility. NO production by iNOS and eNOS can interact with O^2- to form ONOO which can cause LPO, altering ion channel and ion pump function. Cat and GPx are shown as enzymatic pathways to produce water and oxygen from H_2O_2.
Table 2.3 Effect of cyclophosphamide and lipoic acid on the activities of cardiac enzymic antioxidants

<table>
<thead>
<tr>
<th></th>
<th>SOD</th>
<th>CAT</th>
<th>GPx</th>
<th>GR</th>
<th>GST</th>
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<tr>
<td></td>
<td>2.00 ± 0.18</td>
<td>1.32 ± 0.15 a***</td>
<td>2.09 ± 0.23</td>
<td>1.92 ± 0.24 b***</td>
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<td></td>
<td>60.67±5.82</td>
<td>38.83±3.37 a***</td>
<td>61.83±6.68</td>
<td>56.83±5.78 b***</td>
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<td></td>
<td>6.82 ± 0.57</td>
<td>4.18 ± 0.45 a***</td>
<td>6.90 ± 0.62</td>
<td>6.28 ± 0.60 b***</td>
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<td></td>
<td>1.22 ± 0.12</td>
<td>0.59 ± 0.06 a***</td>
<td>1.19 ± 0.13</td>
<td>1.08 ± 0.10 a<em>b</em>**</td>
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<tr>
<td></td>
<td>1.13 ± 0.15</td>
<td>0.74 ± 0.08 a***</td>
<td>1.15 ± 0.15</td>
<td>1.00 ± 0.11 b**</td>
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</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 Groups II, III, IV; b - Group II and Group IV. The symbol ***, ** and * represent statistical significance at $P < 0.001$, $P < 0.01$ and $P < 0.05$ respectively.
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 H₂O₂. Reciprocally SOD may protect CAT and GPx from inhibition by superoxide radicals (Pigeolet et al., 1990). The low levels of enzymic antioxidants and GSH in heart make it vulnerable to free radical damage (Olson et al., 1981). LA administration elevated the activities of SOD, CAT, and GPx by 1.45-, 1.46- and 1.5- fold respectively in Group N when compared to CP-induced Group II animals. LA scavenges hydroxyl radicals, hypochlorous acid, nitric oxide, peroxynitrite, H₂O₂ and singlet oxygen while dihydrolipoic acid (DHLA) scavenges superoxide radical and peroxyl radicals thereby preventing the free radical mediated inactivation of enzymes, restoring them to normalcy (Packer et al., 1995). 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 effusion into the heart, it further exaggerates the already down regulated antioxidant system. LA improves endothelial function (Jones et al., 2002), which in turn reduces protein transudation and therefore enhances the specific activity of antioxidant enzymes.

A significant decline ($P < 0.001$) in the non enzymic antioxidant GSH levels was prominently noted in CP treated rats (Table 2.4)*. Low cardiac GSH levels is a risk factor for developing CP induced congestive heart failure (Dorr and Lagel, 1994). The GSSG/GSH ratio is one of the most important parameters characterizing the prooxidant/antioxidant balance and its increase

* Data published. Please see Annexure.
Table 2.4 Effect of cyclophosphamide and lipoic acid on the cardiac non-enzymic antioxidants

<table>
<thead>
<tr>
<th></th>
<th>Mean ± S.D</th>
<th>Group I vs Group II, III, IV</th>
<th>Group II vs Group IV</th>
<th>Group I vs Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GSH</strong></td>
<td>6.12 ± 0.54</td>
<td>3.71 ± 0.28 <em>a</em>**</td>
<td>6.52 ± 0.49</td>
<td>5.92 ± 0.64 <em>b</em>**</td>
</tr>
<tr>
<td><strong>Vitamin C</strong></td>
<td>1.26 ± 0.16</td>
<td>0.81 ± 0.08 <em>a</em>**</td>
<td>1.35 ± 0.11</td>
<td>1.11 ± 0.15 <em>b</em>**</td>
</tr>
<tr>
<td><strong>Vitamin E</strong></td>
<td>1.03 ± 0.12</td>
<td>0.77 ± 0.08 <em>a</em>**</td>
<td>1.11 ± 0.10</td>
<td>0.94 ± 0.08 <em>b</em>*</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 Groups II, III, IV; b - Group II and Group IV The symbols *** and ** represent statistical significance at $P < 0.001$ and $P < 0.01$ respectively.
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 (Levine et al., 1993). GSH levels were significantly elevated ($P < 0.001$) after LA administration when compared to Group II animals. The redox system, LA/DHLA can regenerate the GSH system (Han et al., 1997). Exogenously supplied LA is rapidly taken up by cells and reduced to DHLA (Packer et al., 1997). Cysteine availability is the rate limiting factor in GSH biosynthesis. DHLA increases cysteine uptake into cell, which is preferentially found in GSH (Estrada et al., 1996).

Significant inhibition ($P < 0.001$) of GSH metabolizing enzymes, GR and GST was noted after CP administration (Table 2.3). 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 sulfhydryl group residues, which are essential for the catalytic activity and are vulnerable to free radicals (Mize et al., 1962). LA administration elevated the activities of GR and GST by 1.83- and 1.35- fold respectively in Group IV when compared to CP induced Group II animals. LA enhanced GR activity indirectly by regenerating the GSH pool. GST detoxifies electrophilic species via an enzyme catalysed conjugation reaction and also plays an active role in detoxification of cytotoxic lipid peroxides (Habig et al., 1974; Ishikawa et al., 1986). 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. A marked decline ($P < 0.001$) in the levels of non enzymic antioxidants vitamins C and vitamin E was noted in CP treated rats (Table 2.4). 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 (Kagan et al., 1992). The restoration of enzymic and non-enzymic antioxidant levels by LA indicates its protective effect against oxidative stress induced by CP.

CP administration resulted in the depletion of cellular thiols, thus indirectly implying an increase in free radicals. A marked drop ($P < 0.001$) in thiols with respect to control group was observed in CP treated rats (Figure 2.8)*. A 1.67-fold decrease in total thiols and a 1.45-fold decrease in nonprotein thiols were observed in Group II. The intracellular thiol levels are accepted to be important in determining the extent of cellular damage induced by chemotherapeutic agents (Mazur and Blawat, 1999). It has been suggested that the reactions of 4-hydroxy cyclophosphamide with various thiol compounds enhance the stability of its metabolites in plasma, as well as facilitate their entry into cells, direct their movement into specific sites inside the cells and delay the release of the alkylating metabolite phosphoramidemustard (Ahmed and Hornbal, 1984). LA restored the cellular thiols thereby protecting cardiac cells from injury. N-acetyl cysteine, another sulphhydryl containing compound has been reported to reduce the myotoxic effects of 4-hydroperoxycyclophosphamide (Dorr and Lagel, 1994). Maintenance of cellular thiol redox also plays a pivotal role in preventing the peroxidation of

* Data published. Please see Annexure.
Figure 2.8  Effect of cyclophosphamide and lipoic acid on thiol levels in the heart tissue

Results are given as mean ± SD for six rats. Comparisons are made between a-Group I and Groups II, III, IV; b - Group II and Group IV. The symbols *** and * represent statistical significance at $P < 0.001$ and $P < 0.05$ respectively.
catalytically essential sulphydryl groups in ATPases (Gardner *et al.*, 1996; Slyshenkov *et al.*, 2002).

### 2.5.5 PEROXIDATIVE CHANGES IN THE MEMBRANES

Gardner *et al.* (1993) have reported that CP administration results in cardiac membrane damage resulting in altered permeability. Alkylation of sulphydryl groups and subsequent crosslinking of ion transporter subunits can prevent the cell from maintaining its physiological ion gradients with the extracellular space leading to cellular injury. The reduction in the activities of ATPases in CP administered animals may be due to enhanced oxidation of membrane lipids and proteins by free radicals and bifunctional crosslinking. Figure 2.9 presents the inhibitory influence of CP on Na⁺,K⁺-ATPases wherein a 33.9% decrease in activity was observed when compared with control group. Moreover cholesterol content is inversely proportional to the membrane fluidity and the activity of Na⁺,K⁺-ATPase in rabbit cardiac muscle cells (Gray *et al.*, 1997). Inhibition of membrane bound Na⁺,K⁺-ATPase will cause an increase in intracellular Na⁺ and loss of K⁺ that leads to membrane depolarization and cardiac arrhythmias (Jacob *et al.*, 1987).

The Na⁺ concentration gradient created by Na⁺,K⁺-ATPase is used to power the export of Ca²⁺ ions by Na⁺/Ca²⁺ antiporter. Disturbances in the ionic gradient will cause a secondary increase in the intracellular calcium through the activity of the antiporter. Ca²⁺-ATPases and Mg²⁺-ATPases exhibited a significant decline (*P* < 0.001) in their activities in Group II (Figure 2.9). Inactivation of Ca²⁺-ATPases and Mg²⁺-ATPases after CP administration corroborate with earlier findings (Wutzen, 1990). Membrane Ca²⁺-ATPase is not active.
Figure 2.9  Effect of cyclophosphamide and lipoic acid on ATPase activities in heart

Results are given as mean ± SD for six rats. Comparisons are made between a - Group I and Groups II, III, IV, b - Group II and Group IV. The symbols *** and * represent statistical significance at $P < 0.001$ and $P < 0.05$ respectively.
responsible for fine tuning of intracellular calcium and is also responsible for the contractility and excitability properties of muscles (Pekiner et al., 2002). Inhibition of Ca\textsuperscript{2+}-ATPases present on sarcoplasmic reticulum may be due to lack of energy which is normally provided by CPK (Levitsky et al., 1978). Decline in the activity of CPK by free radicals may increase the influx of Ca\textsuperscript{2+} from sarcoplasmic reticulum leading to intracellular calcium overload. Alterations in the Mg\textsuperscript{2+} concentration may modulate the activities of Mg\textsuperscript{2+} dependent enzymes, protein synthesis and cell growth (Schulpis et al., 2002). LA treatment substantially improved the activities of ATPases. The dithiol may exert its action by providing its sulphhydryl groups endogenously, which get depleted after CP administration. DHLA scavenges peroxyl radicals in both membrane and aqueous phases. Moreover, LA stabilizes membrane bound enzymes by interacting with vitamin C and glutathione, which may in turn recycle the membrane antioxidant vitamin E (Kagan et al., 1992).

2.5.6 ELECTROLYTE CONTENT

Changes in the extracellular fluid affect the cardiac output and blood pressure. Significant decrease ($P < 0.001$) was observed in the serum levels of calcium and potassium when compared to controls, while increased levels of sodium and magnesium ($P < 0.05$; $P < 0.01$) were evident in CP-induced group (Table 2.5). An increase in norepinephrine in the myocardium caused by CP (Hanaki et al., 1990), may cause hypertension which in turn is associated with hypernatremia (Marzan et al., 2004). It has been reported that hypokalemia poses increased risk of ventricular fibrillation while electrocardiographic conduction abnormalities are common in hypocalcaemia (Diercks et al., 2004).
Table 2.5 Effect of cyclophosphamide and lipoic acid on serum electrolyte levels

<table>
<thead>
<tr>
<th>Electrolyte</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium</td>
<td>141.50 ± 8.07</td>
<td>159.17 ± 10.68 a*</td>
<td>141.85 ± 14.03</td>
<td>142.50 ± 9.48 b*</td>
</tr>
<tr>
<td>Magnesium</td>
<td>1.98 ± 0.26</td>
<td>2.50 ± 0.24 a**</td>
<td>2.03 ± 0.20</td>
<td>2.10 ± 0.23 b**</td>
</tr>
<tr>
<td>Calcium</td>
<td>3.18 ± 0.28</td>
<td>2.19 ± 0.33 a***</td>
<td>3.02 ± 0.28</td>
<td>2.98 ± 0.40 b**</td>
</tr>
<tr>
<td>Potassium</td>
<td>5.38 ± 0.42</td>
<td>3.85 ± 0.37 a***</td>
<td>5.34 ± 0.51</td>
<td>5.29 ± 0.55 b***</td>
</tr>
</tbody>
</table>

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. The symbols ***, ** and * represent statistical significance at $P < 0.001$, $P < 0.01$ and $P < 0.05$ respectively.
Accumulation of intracellular sodium favors an increase in the levels of intracellular calcium due to Na\(^+\)/Ca\(^{2+}\) exchange, and possibly resulting in intracellular calcium overload (Jacob et al., 1987). A significant increase \((P < 0.001)\) in the levels of sodium and calcium with a concomitant decrease \((P < 0.001)\) in potassium and magnesium was noted in cardiac tissue (Table 2.6). Al-Nasser (1998) demonstrated that CP induces cardiotoxicity by increasing heart and liver inner mitochondrial membrane permeability, resulting in calcium efflux. Disruption of the vascular calcium channels can raise cytosolic free calcium levels [Ca\(^{2+}\)], and lead to increased peripheral vascular resistance and hypertension (Vasdev et al., 2002). Levine et al. (1993) speculated that their observed increase in [Ca\(^{2+}\)], decreased K\(^+\)/Na\(^+\) ratio due to both increased [Na\(^+\)], and decreased [K\(^+\)], could be due to a direct effect of 4-HC on the ion transporters. Dietary LA supplementation in spontaneously hypertensive rats lowers the systolic blood pressure and [Ca\(^{2+}\)], (Vasdev et al., 2000).

Stress increases the membrane permeability of catecholamine sensitive cells, which in turn raises calcium influx into cells and liberates intracellular magnesium. Magnesium is involved in all physiological interactions in myocardial tissue, coronary artery smooth muscle and sarcolemma conducting systems (Mahboob et al., 1996). Both magnesium and potassium are predominant intracellular cations. Potassium plays an important role in maintaining the electric potential across the cellular membrane as well as in depolarisation and repolarisation of the myocytes. Abnormalities could arise from the mechanism of potassium regulation involving active transport through Na\(^+\),K\(^+\)-ATPase, insulin, beta adrenergic agents, mineralocorticoids
Table 2.6 Effect of cyclophosphamide and lipopolysaccharide on electrolyte levels in heart tissue

<table>
<thead>
<tr>
<th></th>
<th>Sodium</th>
<th>Magnesium</th>
<th>Calcium</th>
<th>Potassium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>41.83 ± 4.49</td>
<td>54.17 ± 5.31 a***</td>
<td>42.01 ± 4.34</td>
<td>43.83 ± 4.79 b**</td>
</tr>
<tr>
<td></td>
<td>5.82 ± 0.48</td>
<td>3.87 ± 0.48 a***</td>
<td>5.72 ± 0.40</td>
<td>5.62 ± 0.53 b***</td>
</tr>
<tr>
<td></td>
<td>1.92 ± 0.17</td>
<td>2.95 ± 0.26 a***</td>
<td>2.02 ± 0.29</td>
<td>2.18 ± 0.21 b***</td>
</tr>
<tr>
<td></td>
<td>74.67 ± 6.77</td>
<td>60.83 ± 6.94 a***</td>
<td>74.17 ± 7.78</td>
<td>73.33 ± 6.47 b**</td>
</tr>
</tbody>
</table>

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.

The symbols *** and ** represent statistical significance at P < 0.001 and P < 0.01 respectively.
and passive transport (Dieroks et al., 2004). LA efficiently prevented the electrolyte abnormalities in CP induced rats.

2.5.7 NITROSATIVE STRESS

NO (Nitric oxide) is a free radical with an unpaired electron (Akiyama et al., 1998). It is a small signaling molecule generated by a family of nitric oxide synthases (NOS), which convert L-Arginine to NO and L-citrulline (Stuehr, 1999). In the normal heart, NO is synthesized by constitutive Ca\(^{2+}\)-dependent NO synthases in cardiac myocytes, vascular and endocardial endothelium (NOS III or eNOS) as well as in specific cardiac neurons (NOS I or nNOS) and plays an important role in the regulation of coronary circulation and cardiac contractile function (Casadei and Sears, 2003). The calcium independent inducible form (NOS II or iNOS) is expressed in cardiomyocytes and inflammatory cells infiltrating the myocardium under stress conditions (Jung et al., 2000).

Nitric oxide (NO) plays diverse roles in cardiac function and disease. Many of the cardiac actions of NO appear to be mediated through elevation of intracellular cGMP content, secondary to the activation of soluble guanyl cyclase upon binding of NO to the heme moiety of the enzyme (Shah and MacCarthy, 2000). Basal production of NO via constitutive NOS isoforms modulates cardiomyocyte contractility and regulates blood flow distribution and acts as a potent vasodilator (Varin et al., 1999; Yan et al., 2001). In contrast, high levels of NO production via iNOS are associated with several forms of cardiac disease, including dilated cardiomyopathy and congestive heart failure (Haywood et al., 1996). The high concentrations of NO achieved through iNOS induction may participate in further cardiomyocyte oxidative
damage, apoptosis, and/or necrosis (Aldieri et al., 2002). The levels of NO metabolites, nitrate and nitrite increased 1.94-fold in the plasma of CP administered rats (Figure 2.10). The appearance of oxidative products of NO (NO$_2^-$ and NO$_3^-$) referred to as NO$_x$, in peripheral blood of patients with acute MI is the result of their increased release from infarcted heart during the inflammatory phase of myocardial ischemia (Akiyama et al., 1998). NO-induced inhibition of respiration results from a reversible inhibition of cytochrome oxidase by NO itself, and an irreversible inhibition of multiple mitochondrial components by peroxynitrite or S-nitrosothiols (Cassina and Radi, 1996).

Peroxynitrite (ONOO$^-$) is a highly cytotoxic oxidant formed in the nearly instantaneous reaction of NO with superoxide anion (Radi et al., 1991). Mihm et al. (2001) found that the cardiac myofibril is a primary site of protein nitration during adriamycin cardiotoxicity and that this contractile structure is highly sensitive to ONOO$^-$ mediated injury, particularly the myofibrillar isoform of creatine kinase. The cytotoxic effects of ONOO$^-$ include nitration of tyrosine residues to form 3-nitrotyrosine and oxidative DNA damage leading to tissue injury (Yermilov et al., 1995; Kanski et al., 2003). Increased expression of iNOS mRNA in heart tissue was evident in heart tissue of CP treated rats (Figure 2.11). RT-PCR analysis of iNOS mRNA showed a significant ($P < 0.001$) increase in CP administered rats (Figure 2.12). In previous studies, apoptosis and iNOS immunoreactivity were notable in the damaged urothelium of rats administered CP (Jezernik et al., 2003). Xu et al. (2001) observed that CP administration increased NO$_x$ levels in the urine and plasma of rats. Incubation of primary cell cultures of rat bladder smooth muscle with plasma from rats treated with CP increased the
Figure 2.10  Effect of cyclophosphamide and lipoic acid on $\text{No}_x$ levels in plasma

Results are given as mean ± SD for six rats. Comparisons are made between a - Group I and Groups II, III, IV, b - Group II and Group IV. The symbols *** and * represent statistical significance at $P < 0.001$ and $P < 0.05$ respectively.
Figure 2.11  Effect of cyclophosphamide and lipote acid on expression of FvO5 mRNA in heart tissue

Lane 1: DNA 100 bp ladder
Lane 2: Control
Lane 3: CP
Lane 4: LA
Lane 5: CP + LA
Figure 2.12  Relative levels of iNOS mRNA expression compared to RPL-19 after cyclophosphamide and lipoic acid administration

Results are given as mean ± SD for six rats. Comparisons are made between a – Group I and Groups II, III, IV, b – Group II and Group IV. The symbols *** represents statistical significance at $P < 0.001$.
iNOS expression and NO production. They reported that increased production of NO and circulatory factors could have triggered the increased iNOS expression. Both LA and DHLA are known to scavenge NO and ONOO⁻ radicals (Packer et al., 2001). LA suppressed the expression of iNOS and decreased the levels of NO₂ in Group IV. This corroborates with an earlier report where LA reversed the upregulation of iNOS mRNA expression during hyperglycemia (Bojunga et al., 2004).

2.5.8 DESTABILISATION OF LYSOSOMES

Lysosomes are essential for controlled intracellular digestion of cellular components by different pathways such as autophagy, heterophagy, and endocytosis. Depending on the state of their operative digestive function, lysosomal structures are generally classified into primary lysosomes, those that have not yet encountered materials to be digested, or secondary lysosomes, those that are engaged in overt digestive activity (Yamamoto et al., 2000). Considerable attention has been focused on lysosomal alterations that might accompany ischemic or hypoxic myocellular damage.

The localization of acid hydrolases in cardiac myocytes, is in the lysosomes and the release of these enzymes from the lysosome to the cytosol leads to myocardial cellular injury and death in the ischemic state of the heart (Ricciutti, 1972; Decker and Wildenthal, 1980). This is in agreement with our findings showing that in CP administered rats the activities of serum lysosomal acid hydrolases, β-Glu, β-Gal, NAG, Cat-D and ACP, increased significantly ($P < 0.001$) when compared to controls (Table 2.7). Cathepsins are lysosomal proteases possibly involved in autophagic digestion of discrete areas of cytoplasm and myofibrillar and mitochondrial proteins (Zak et al.,
Table 2.7 Effect of cyclophosphamide and lipoic acid on the activities of lysosomal hydrolases in serum

<table>
<thead>
<tr>
<th></th>
<th>β-Glu</th>
<th>β-Gal</th>
<th>NAG</th>
<th>Cat-D</th>
<th>ACP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10.83 ± 1.60</td>
<td>16.02 ± 2.12 a***</td>
<td>11.17 ± 1.72</td>
<td>12.50 ± 1.52 b**</td>
<td></td>
</tr>
<tr>
<td>β-Gal</td>
<td>15.67 ± 1.97</td>
<td>23.50 ± 1.87 a***</td>
<td>15.92 ± 1.86</td>
<td>16.50 ± 1.90 b***</td>
<td></td>
</tr>
<tr>
<td>NAG</td>
<td>16.33 ± 1.86</td>
<td>24.83 ± 2.64 a***</td>
<td>16.75 ± 2.04</td>
<td>17.33 ± 1.75 b***</td>
<td></td>
</tr>
<tr>
<td>Cat-D</td>
<td>13.02 ± 1.56</td>
<td>19.02 ± 1.90 a**</td>
<td>13.67 ± 1.63</td>
<td>15.10 ± 1.99 b**</td>
<td></td>
</tr>
<tr>
<td>ACP</td>
<td>71.17 ± 6.31</td>
<td>113.83 ± 9.28 a***</td>
<td>71.97 ± 7.69</td>
<td>73.67 ± 8.57 b***</td>
<td></td>
</tr>
</tbody>
</table>

Results are given as mean ± S.D for six rats. Activities are expressed as: β-GLU, β-GAL and NAG: μmoles x 10⁻² of p-nitrophenol liberated/h/mg protein; Cat-D: μmoles x 10⁻² of tyrosine liberated/h/mg protein; ACP μmoles x 10⁻² of phenol released/h/mg protein. Comparisons are made between: a - Group I and Groups II, III, IV; b - Group II and Group IV. The symbols *** and ** represent statistical significance at P < 0.001 and P < 0.01 respectively.
1976). Ravens and Gudbjarnason (1969) observed that the release of hydrolytic enzymes from lysosomes after coronary occlusion may be a causative factor for the development of myocardial cellular destruction.

The phospholipid-rich lysosomal membrane is a potential site of free radical attack subsequently causing loss of membrane stability. Kalra and Prasad (1994) have suggested that ROS generated during ischemia, in addition to the direct myocardial damaging effect, may also be responsible for the cardiac damage through the release of lysosomal enzymes. Apoptosis or programmed cell death which follows from moderate oxidative stress is preceded by partial lysosomal rupture and such lysosomal destabilization seems to be an initial event also in apoptosis caused by a variety of other agents. The way in which this destabilization may lead to apoptosis is not yet clear but may involve processes such as activation of procaspases or other proapoptotic proteins by lysosomal proteases, or attack on mitochondrial and lysosomal membranes by lysosomal constituents (Brunk et al., 2001).

Secondary lysosomes were prominent near degenerative intracellular organelles in both hypertrophic and atrophic cardiocytes (Yamamoto et al., 2000). The compensatory hypertrophic changes after CP administration (Hopkins et al., 1982) corroborate with an abnormal increase in the activities of lysosomal enzymes in cardiac tissue (Table 2.8). The activities of cardiac lysosomal acid hydrolases, β-Glu, β-Gal, NAG, Cat-D and ACP, increased by 1.47-, 1.32-, 1.29-, 1.66- and 1.21- fold respectively (Table 2.8). Earlier experimental results with LA and α-lipoamide suggested that both were able to suppress cell killing by oxidants through stabilizing lysosomes. Cell death caused by oxidants may be initiated by lysosomal rupture and that this latter
Table 2.8 Effect of cyclophosphamide and lipoic acid on the activities of lysosomal hydrolases in heart tissue

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td><strong>β-Glu</strong></td>
<td>25.67 ± 2.88</td>
<td>37.92 ± 4.22 a***</td>
<td>25.92 ± 3.11</td>
<td>26.17 ± 2.23 b**</td>
</tr>
<tr>
<td><strong>β-Gal</strong></td>
<td>37.17 ± 3.66</td>
<td>49.17 ± 6.27 a***</td>
<td>37.83 ± 3.31</td>
<td>40.58 ± 4.80 b**</td>
</tr>
<tr>
<td><strong>NAG</strong></td>
<td>40.83 ± 3.06</td>
<td>52.67 ± 4.72 a***</td>
<td>41.17 ± 3.76</td>
<td>42.17 ± 4.22 b***</td>
</tr>
<tr>
<td><strong>Cat-D</strong></td>
<td>21.17 ± 2.32</td>
<td>35.17 ± 3.25 a***</td>
<td>21.83 ± 2.04</td>
<td>24.67 ± 2.94 a<em>b</em>**</td>
</tr>
<tr>
<td><strong>ACP</strong></td>
<td>112.83 ± 9.37</td>
<td>136.83 ± 9.93 a***</td>
<td>112.17 ± 7.17</td>
<td>113.83 ± 8.73 b***</td>
</tr>
</tbody>
</table>

Results are given as mean ± S.D for six rats. Activities are expressed as: **β-GLU**, **β-GAL** and **NAG**: μmoles × 10⁻² of p-nitrophenol liberated/h/mg protein; **Cat-D**: μmoles × 10⁻² of tyrosine liberated/h/mg protein; **ACP** μmoles × 10⁻² of phenol released/h/mg protein. Comparisons are made between: a - Group I and Groups II, III, IV; b - Group II and Group IV. The symbols ****, ** and * represent statistical significance at P < 0.001, P < 0.01 and P < 0.05 respectively.
event may involve intralysosomal iron which catalyzes Fenton-type chemistry and resultant peroxidative damage to lysosomal membranes. The vicinal thiols present in the reduced forms of these compounds suggest that the antioxidant actions may arise from intralysosomal iron chelation (Persson et al., 2003). The increase in the activities of lysosomal enzymes leads to altered metabolism of different connective tissue constituents namely glycosaminoglycan, glycoprotein (Mathew et al., 1982) and collagen (Takahashi et al., 1990) in experimentally induced myocardial infarction.

2.5.9 ELEVATION OF HYDROXYPROLINE CONTENT INDICATING FIBROSIS

The cardiac muscle reveals significant changes in cellular composition and deposition of extracellular matrix in heart failure. A 1.62-fold increase in hydroxyproline was observed in Group II CP treated rats (Figure 2.13). Hydroxyproline is a classic marker of fibrosis. Increase in hydroxyproline content indicating fibrosis in the cardiac tissue of CP treated rats has been reported earlier (Hopkins et al., 1982). The increased left ventricular diameter is caused by apoptotic and necrotic loss of cardiomyocytes and by increased deposition of collagen, resulting in increased fibrosis of the ventricular wall (Milting et al., 2003). Diffuse left and right ventricular fibrosis, and ischemia impart increased risk and are the major underlying mechanisms in impaired myocytic function during hypertrophy, commonly termed as cardiac enlargement (Frohlich, 2001) (Figure 2.14). Increase in biosynthesis of collagen measured by the formation of $^3$H hydroxyproline and increase in lung hydroxyproline after CP treatment has been reported earlier (Venkatesan
Figure 2.13  Effect of cyclophosphamide and lipoic acid on hydroxyproline levels in heart tissue

Results are given as mean ± SD for six rats. Comparisons are made between: a - Group I and Groups II, III, IV; b - Group II and Group IV. The symbols ***, * represent statistical significance at $P < 0.001$, $P < 0.05$ respectively.
et al., 1998). In Group IV, LA supplementation substantially reduced the hydroxyproline levels.

Figure 2.14 Fibrosis as the physiopathologic protagonist in cardiovascular syndromes

2.5.10 MITOCHONDRIAL DERANGEMENTS

The free radicals produced after CP administration may also affect the mitochondria which are vital for the proper function of the adult myocardium to fulfill its function as a circulatory pump and to maintain ion homeostasis. Alkylating agents such as CP rapidly impair cellular respiration (Souid et al., 2003) and also damage the heart inner mitochondrial membrane. CP-induced cardiotoxicity may be due to heart inner mitochondrial membrane permeability to calcium ions (Al Nasser, 1998). Microthrombosis occurring after CP administration (Goldberg et al., 1984) may affect tissue supply with oxygen and substrates, which may reflect in the diminished mitochondrial function (Zorov et al., 2005).

The mitochondrial enzymes catalyze the oxidation of several substrates through the TCA cycle, yielding reducing equivalents which are channeled through the respiratory chain for the synthesis of ATP by oxidative phosphorylation. The activities of ICDH, SDH and MDH decreased
significantly by 38.41%, 31.24% and 41.2% respectively after CP administration in Group II when compared to control Group (Figure 2.15)*. Inhibition of these enzymes by ROS may affect the mitochondrial substrate oxidation, resulting in reduced oxidation of substrates, reduced rate of transfer of reducing equivalents to molecular oxygen and depletion of cellular energy. Mitochondrial dysfunction, reflected in the structure, function and number of mitochondria within the cardiomyocyte (Capetanaki, 2002) may be responsible for the diminished activities of these enzymes.

The enzyme NADP dependent ICDH is mainly expressed in the heart and skeletal muscle mitochondria (Plaut et al., 1983). It controls the mitochondrial redox balance and the subsequent oxidative damage. ICDH restores NADPH that in turn regenerates reduced glutathione (GSH) by glutathione reductase and is critical for the activity of NADPH-dependent thioredoxin reductase and catalase (Lee et al., 2002). Decline in the ICDH activity may be attributed to CP-induced loss of mitochondrial calcium which is required for the stimulation of dehydrogenases (Rosenberg, 2004). In addition to its role in the TCA cycle, SDH is a component of the electron transport chain and is bound to the inner mitochondrial membrane. It loses its activity when vicinal thiols groups are oxidized (Le-Quoc et al., 1981). Increase in the activities of TCA cycle enzymes in LA treated animals indicates better utilization of energy yielding intermediates by TCA cycle.

The mitochondrial respiratory chain is the major source of superoxide and therefore mitochondria are more susceptible to oxidative damage than the rest of the cell, contributing to mitochondrial dysfunction (Lenaz, 1998).

* Data published. Please see Annexure.
Figure 2.15  Effect of cyclophosphamide and lipoic acid on the activities of TCA cycle enzymes in heart

Results are given as mean ± SD for six rats. Units: ICDH: nmole of NADPH formed/min/mg protein; SDH: nmole of succinate oxidized/min/mg protein; MDH: nmole of NADH oxidized/min/mg protein. Comparisons are made between: a - Group I and Groups II, III, IV; b - Group II and Group IV. The symbols *** and * represent statistical significance at $P < 0.001$ and $P < 0.05$ respectively.
Statistically significant ($P < 0.001$) decrease in the activities of Complex I, II, III and IV was noted in Group II CP treated rats (Table 2.9). Decline in the activities of respiratory complexes resulting in impaired oxidative phosphorylation could be due to CP-induced oxidative damage to mitochondrial DNA (Branda et al., 2002) which codes for several proteins including complexes I, III and IV (Antozzi and Zeviani, 1997). The ROS generated by CP may also inactivate the iron sulphur centers of complexes I, II and III (Drouet et al., 1999).

NADH dehydrogenase, a flavin linked dehydrogenase passes electrons from NADH to Coenzyme Q. The decrease in its activity may be due to the depletion of reducing equivalents like NADH and NADPH that are utilized for the formation of GSH to counter oxidative damage of mitochondrial components (Singh, 2002). Impairment in the activities of complexes III and IV may be due to the peroxidation of cardiolipin, a phospholipid present in mitochondrial membrane (Paradies et al., 2001). Inactivation of cytochrome c oxidase after CP treatment (Sudharsan et al., 2006b) is consistent with an earlier report which could be due to CP-induced membrane LPO (Gvozdjakova et al., 1982). Decline in cytochrome c oxidase activity can cause an increase in $H_2O_2$ production. It may be speculated that decline in cytochrome c oxidase activity can result in partial blockage of electron flow, which alters reducing potentials of some electron carriers favoring their autoxidation, electron leak and consequent generation of superoxide (Sohal et al., 1993). The defects in cardiac energy metabolism may cause heart failure (Figure 2.16).

* Data published. Please see Annexure.
Table 2.9 Effect of cyclophosphamide and lipoic acid on the activities of respiratory chain enzymes in heart

<table>
<thead>
<tr>
<th>Complex</th>
<th>Control</th>
<th>Cyclophosphamide</th>
<th>Lipoic acid</th>
<th>Combined Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex I</td>
<td>1.92 ± 0.17</td>
<td>1.33 ± 0.16 a***</td>
<td>1.87 ± 0.24</td>
<td>1.75 ± 0.14 b***</td>
</tr>
<tr>
<td>Complex II</td>
<td>0.97 ± 0.15</td>
<td>0.62 ± 0.10 a***</td>
<td>0.99 ± 0.16</td>
<td>0.90 ± 0.12 b**</td>
</tr>
<tr>
<td>Complex III</td>
<td>2.12 ± 0.21</td>
<td>1.25 ± 0.15 a***</td>
<td>2.18 ± 0.24</td>
<td>1.97 ± 0.22 b***</td>
</tr>
<tr>
<td>Complex IV</td>
<td>3.60 ± 0.26</td>
<td>2.30 ± 0.24 a***</td>
<td>3.58 ± 0.31</td>
<td>3.47 ± 0.29 b***</td>
</tr>
</tbody>
</table>

Results are given as mean ± S.D for six rats. Units: Complex I: μmoles of NADH oxidized/min/mg protein; Complex II: μmoles of DCIP reduced/min/mg protein; Complex III: μmoles of cytochrome c reduced/min/mg protein; Complex IV: μmole of cytochrome c oxidized/min/mg protein. Comparisons are made between: a - Group I and Groups II, III, IV; b - group II and group IV. The symbols *** and ** represent statistical significance at $P < 0.001$ and $P < 0.01$ respectively.
LA was effective in improving the cardiac mitochondrial function after CP administration. Exogenously supplied LA is rapidly taken up by cardiac mitochondria and reduced to the powerful antioxidant DHLA by lipoamide dehydrogenase (Haramaki et al., 1997). DHLA causes a remarkable suppression of mitochondrial superoxide production in rat heart. It penetrates phospholipid membranes and interferes with the radical generating machinery of redox-cycling ubisemiquinone and thus reduces superoxide release (Schonheit et al., 1995). LA acts as a cofactor for the multienzyme complexes (Packer et al., 1995), scavenges free radicals at their mitochondrial source (Biewenga et al., 1997) and augments the cellular thiols. It is known to improve mitochondrial function by reducing oxidative stress (Saada et al., 2004).

2.5.11 HYPERLIPIDEMIC CARDIOMYOPATHY

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 CP treated animals suggest that these adducts may play a role in the development of atherosclerosis or atherogenesis (Arikketh et al., 2004). CP is known to result in hypertriglyceridemia and hypercholesterolemia, which are well known risk factors in cardiovascular diseases (Loudet et al., 1984).

A significant increase in the levels of free and esterified or storage form of cholesterol \((P < 0.01)\) was observed in serum and heart of CP treated rats when compared to controls (Tables 2.10 and 2.11). These changes could be due to increase in the biosynthesis and decrease in its utilization. CP induces free radicals, which cause cellular cholesterol accumulation, (a) by increasing cholesterol biosynthesis and its esterification, (b) by decreasing cholesteryl ester hydrolysis (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 CP (200 mg/kg b.wt) 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 tissue phospholipid content with a concomitant increase in the serum phospholipid 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). Similar decrease in cardiac phospholipid levels was observed after CP administration in rats (Sudharsan et al., 2005b).
Table 2.10 Effect of cyclophosphamide and lipoic acid on serum lipids

<table>
<thead>
<tr>
<th></th>
<th>38.17 ± 4.71</th>
<th>61.67 ± 5.20 a***</th>
<th>37.33 ± 3.78</th>
<th>40.83 ± 5.19 b***</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free Cholesterol</td>
<td>49.50 ± 4.37</td>
<td>78.17 ± 5.95 a***</td>
<td>49.83 ± 4.71</td>
<td>54.17 ± 4.62 b***</td>
</tr>
<tr>
<td>Esterified Cholesterol</td>
<td>101.33 ± 8.52</td>
<td>148.67 ± 11.33 a***</td>
<td>101.02 ± 8.35</td>
<td>104.07 ± 9.51 b***</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>13.85 ± 1.71</td>
<td>28.83 ± 2.14 a***</td>
<td>13.67 ± 1.37</td>
<td>16.58 ± 1.80 a<em>b</em>**</td>
</tr>
<tr>
<td>Free Fatty Acids</td>
<td>67.33 ± 6.25</td>
<td>104.50 ± 9.16 a***</td>
<td>67.67 ± 7.28</td>
<td>74.67 ± 6.09 b***</td>
</tr>
</tbody>
</table>

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. The symbols *** and * represent statistical significance at $P < 0.001$ and $P < 0.05$ respectively.
Table 2.11 Effect of cyclophosphamide and lipoic acid on cardiac lipid status

<table>
<thead>
<tr>
<th></th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free Cholesterol</td>
<td>3.45 ± 0.42</td>
<td>4.22 ± 0.46 a**</td>
<td>3.40 ± 0.39</td>
<td>3.55 ± 0.40 b*</td>
</tr>
<tr>
<td>Esterified Cholesterol</td>
<td>1.82 ± 0.26</td>
<td>2.85 ± 0.39 a***</td>
<td>1.80 ± 0.22</td>
<td>1.92 ± 0.28 b***</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>13.83 ± 1.47</td>
<td>9.50 ± 1.87 a***</td>
<td>14.02 ± 1.67</td>
<td>12.98 ± 1.82 b*</td>
</tr>
<tr>
<td>Free Fatty Acids</td>
<td>2.98 ± 0.23</td>
<td>1.98 ± 0.25 a***</td>
<td>3.02 ± 0.19</td>
<td>2.85 ± 0.25 b***</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>4.53 ± 0.53</td>
<td>7.43 ± 0.96 a***</td>
<td>4.55 ± 0.46</td>
<td>4.82 ± 0.35 b***</td>
</tr>
</tbody>
</table>

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. The symbols ***, ** and * represent statistical significance at $P < 0.001$, $P < 0.01$ and $P < 0.05$ respectively.
Cholesterol and phospholipids 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. In plasma, VLDL is degraded into IDL and LDL by the action of 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. In the present study, a significant decrease ($P < 0.001$) in HDL cholesterol was associated with an increase in LDL and VLDL ($P < 0.001$) in CP treated rats (Figure 2.17). Previously in CP treated rats, lipid composition showed that HDL cholesterol was very low comparatively to a high VLDL cholesterol (Loudet et al., 1984). VLDL was larger than normal, corresponding to triglyceride enrichment (Lespine et al., 1988).

Triacylglycerols are degraded by the enzyme 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 hypertriglyceridaemia during CP treatment. Simultaneously heart LPL activity was also decreased in fasted animals (Lespine et al., 1997). Due to the decrease ($P < 0.001$) in LPL activity (Table 2.12), increase in triglycerides was associated with a drop in fatty acid levels in the heart of Group II CP treated rats. The moderate increase in the rate of triacylglycerol synthesis by the liver contributes to the occurrence of hypertriglyceridaemia in CP-treated rats (Lespine et al., 1993). Hypercholesterolemic changes in these rats may be explained by a marked reduction in the activities of fat splitting enzymes, such as plasma LCAT.
Figure 2.17  Effect of cyclophosphamide and lipoic acid on serum lipoprotein fractions

Results are given as mean ± SD for six rats. Comparisons are made between: a – Group I and Groups 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 2.12 Effect of cyclophosphamide and lipoic acid on the activities of lipid metabolizing enzymes

<table>
<thead>
<tr>
<th></th>
<th>Heart</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CES</td>
<td>9.05 ± 0.84</td>
<td>14.25 ± 1.72 a***</td>
<td>9.03 ± 1.17</td>
<td>10.58 ± 1.02 a<em>b</em>**</td>
</tr>
<tr>
<td>CEH</td>
<td>15.08 ± 1.50</td>
<td>11.08 ± 1.43 a***</td>
<td>15.02 ± 1.41</td>
<td>14.08 ± 1.86 b***</td>
</tr>
<tr>
<td>LPL</td>
<td>13.42 ± 1.02</td>
<td>8.97 ± 1.28 a***</td>
<td>13.27 ± 1.18</td>
<td>12.90 ± 1.91 b***</td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LCAT</td>
<td>8.18 ± 0.58</td>
<td>5.45 ± 0.54 a***</td>
<td>8.22 ± 0.75</td>
<td>7.67 ± 0.79 b***</td>
</tr>
</tbody>
</table>

Results are given as mean ± S.D for six rats. Units of enzyme activity: CES: nmols of cholesterol esterified/h/mg protein at 37°C; CEH: nmols of cholesterol liberated/h/mg protein at 37°C; LPL: µmoles of free fatty acids liberated/h/mg protein at 37°C; LCAT: µmoles 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. The symbols *** and * represent statistical significance at $P < 0.001$ and $P < 0.05$ respectively.
(P < 0.001) and cardiac LPL (P < 0.001) (Table 2.12). 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). An increase (P < 0.01) in the activity of CES, with a significant decrease (P < 0.001) in the activity of CEH, was observed in cyclophosphamide-treated rats when compared to controls (Table 2.12). 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.

Treatment with LA starting with the high cholesterol diet, limited the diet-dependent increase of lipids in the plasma, liver, and aorta (Angelucci and Mascitelli-Coriandoli, 1958). Evidence has also been reported that i.v. treatment of patients with LA improves the process of lipid catabolism as well as induces an increase in the serum protein content, suggesting an action of LA at a common step in the metabolic process of degradation and synthesis of lipids and proteins (Bustamante et al., 1998; Gallone and Consolo, 1957). Antioxidants such as LA could be beneficial in countering cholesterol accumulation by scavenging free radicals. LA 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 LA on both lipid and hemostatic risk factors are of particular importance in cardiovascular diseases.
2.5.12 MORPHOLOGICAL CHANGES-CONFIRMATION AND CORRELATION WITH BIOCHEMICAL FINDINGS

The cardiac toxicity induced by CP is further confirmed by the abnormal histologic findings in the heart of CP exposed animals*. Animals treated with CP (Group II) show abnormal cardiac muscle with karyorrhexis, pycnotic nuclei and extensive intermuscular haemorrhage (Figure 2.18b). These findings corroborate with previous reports where multiple areas of myocardial haemorrhage have been identified after CP administration (Buja et al., 1976). The control Groups I and III present cardiac muscle fibers with normal architecture (Figure 2.18a and 2.18c). LA treated Group IV photomicrograph shows cardiac fibers exhibiting significant recovery from CP-induced cytotoxic damage (Figure 2.18d). These findings thereby highlight the cytoprotective role of LA in ameliorating the cardiac injury caused by CP.

* Findings published. Please see Annexure.
Figure 2.19 Histopathology of heart tissues in the four experimental groups (H&E, 200 x)

- (a) Control group shows normal architecture.
- (b) CF induced Group II shows disorganized cardiac muscle with hypertrophic, pyknotic nuclei and extensive interstitial hemorrhage.
- (c) LA drug control group exhibits normal architecture.
- (d) LA treatment in CF induced Group IV shows a significant recovery in the cardiac fibrous.