ABSTRACT

The Protective Effect of α-Lipoic Acid in Doxorubicin Induced Cardiotoxicity in Rats

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This study examined the protective effects of α-lipoic acid in rats exposed to a cardiotoxic dose of the widely used chemotherapy agent doxorubicin. α-Lipoic acid significantly enhanced the survival of rats exposed to a lethal dose of doxorubicin ($p<0.001$). Morbidity was proportionally higher in rats treated with doxorubicin alone in contrast to rats treated with both doxorubicin and α-lipoic acid ($p<0.001$). Heart weights of rats treated with both α-lipoic acid and doxorubicin were similar to that of control rats, and controlling for whole body weight yielded similar results ($p<0.001$). α-Lipoic acid significantly reduced the cardiac microscopic damage caused by doxorubicin in rats ($p<0.001$). Representative micrographs supported a high degree of ultrastructural preservation in doxorubicin treated rats upon concomitant treatment with α-lipoic acid. The conclusion of this study is that α-lipoic acid is effective in protecting rats from doxorubicin induced cardiotoxicity.

The Protective Effect of α-Lipoic Acid in Doxorubicin Induced Cardiomyopathy in Rats

by

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CHAPTER ONE

Introduction

Currently, cancer treatment options include chemotherapy, surgery, and radiation treatments. Antineoplastic agents offer help to cancer patients with varying degrees of success, with doxorubicin being very potent against a large spectrum of malignancies (Arcamone, 1985).

Chemotherapy regimens offer many cancer patients a tumorcidal benefit. Unfortunately, these treatments may put the patient at risk of life threatening complications. Many cancer patients face chemotherapy induced cardiotoxicity, especially when doxorubicin is used. Cardiotoxicity is a nonreversible doxorubicin side effect, and it adversely affects overall treatment outcomes. Many variables are involved in the toxicity, including dosage level, the patient's age, combination therapy with other chemotherapeutics, and concurrent radiation treatment (Watts, 1991). This leads to restriction of chemotherapy dosages and necessitates strict physiological surveillance on patients (Hortobagyi, 1997). Efforts are underway to make chemotherapy drugs safer, including targeted delivery approaches, and thus allowing oncologists to administer safer tumorcidal doses to their patients.

A promising approach in chemotherapy research aims to elucidate the mechanism of side effects in an effort to control them, thereby enhancing the success and safety of chemotherapy treatment. In particular, researchers are continually investigating a variety of compounds in hopes of mitigating cardiotoxicity induced by doxorubicin. This study attempts to modulate doxorubicin induced cardiotoxicity in rats using α-lipoic acid.

CHAPTER TWO

Literature Review

Doxorubicin

The anthracycline doxorubicin, also known as adriamycin, is an antibiotic isolated from *Streptomyces* species. Doxorubicin is one of the most important antitumor agents, having a broad spectrum of therapeutic potency against a variety of human tumors including soft tissue sarcoma, breast cancer, small cell carcinoma of the lung, and acute leukemias (Arcamone, 1985).

Antitumor Activity

The anthracycline doxorubicin binds covalently to DNA in tumor cells, thereby disrupting cell replication and metabolism. This seems to be the primary mode of antitumor action, although damage to other biological macromolecules cannot be excluded as a secondary cytotoxic mechanism (Taatjes et al., 1999).

Toxic Side Effects

Doxorubicin displays toxic effects against a variety of cells; its cardiotoxicity is partially due to the propensity of cardiac muscle to accumulate doxorubicin. This places a limit on the total dose that may be given, since the effect is cumulative over several months (Goormaghtigh and Ruysschaert, 1984). Irreversible congestive heart failure has been documented in greater than thirty percent of patients after treatment with cumulative doses of doxorubicin above 600 mg/m^2 (Minow et al., 1975). Doxorubicin treated patients are at risk for acute and chronic cardiotoxicities, imposing dose restrictions and

surveillance of cardiovascular performance. Other toxic effects of doxorubicin, including stomatitis, nausea, vomiting, and alopecia, are generally reversible (Hortobagyi, 1997).

Risk factors for doxorubicin induced cardiomyopathy include age, dosing schedule, thoracic irradiation, general anesthesia, multiagent therapy, and poor nutrition. Three cases of pediatric congestive heart failure directly were related to cumulative doxorubicin doses of less than 400 mg/m^2. Two of the patients died at the time of publication in 1991. The patients were receiving multi-agent therapy for osteogenic or synovial cell sarcoma at the onset of heart failure (Watts, 1991).

Doxorubicin Mediated Oxidative Damage

The anthracycline antibiotic doxorubicin is known as a redox cycling drug, because it shuttles electrons from donor(s) to acceptor(s) by virtue of its continual reduction-reoxidation. For example, the microsomal flavoenzyme NADPH-cytochrome P450 reductase catalyzes a one electron reduction of doxorubicin to a semiquinone free radical ($DOX^{\cdot}$), which in turn regenerates the parent compound by auto-oxidizing at the expense of molecular oxygen. Doxorubicin stimulates microsomal oxidation of NADPH and formation of superoxide radical, the latter is dismutated into hydrogen peroxide by superoxide dismutase, a component of the natural cellular defense against free radicals. Intracellular iron, free or ferritin bound, plays the next critical role in mediating the steps of oxidative damage. Intracellular iron can be viewed as a double-edged sword; some iron is necessary for cell functions including respiration and DNA synthesis, however, excess or imbalanced iron catalyzes free radical reactions that damage polyunsaturated fatty acids, proteins, and nucleic acids. In biological systems, ferric iron Fe (III) is reduced by superoxide to ferrous iron Fe (II) in a process known as iron catalyzed Haber-

Weiss reaction. The resulting Fe (II) catalyzes cleavage of peroxide to highly reactive peroxyl radical $OH^{\cdot}$ through the Fenton reaction. The above reactions are both referred to as the superoxide driven Fenton reaction.

$$O_2^{\cdot}- + Fe\ (III) \rightarrow O_2 + Fe\ (II) \quad \text{.....[1]}$$

$$2O_2^{\cdot}- + 2H{+}{+} \rightarrow H_2O_2 + O_2 \quad \text{.....[2]}$$

$$Fe\ (II) + H_2O_2 \rightarrow Fe\ (III) + OH- + OH^{\cdot} \quad \text{.....[3]}$$

It is known that $OH^{\cdot}$ abstracts hydrogen from the *bis*-allylic bond of polyunsaturated phospholipids, yielding an alkyl radical and causing rearrangement of double bonds in the form of a conjugated diene. Subsequent reaction of alkyl radicals with molecular oxygen generates peroxyl radicals that abstract hydrogen from a neighboring allylic bond, yielding LOOH and a new conjugated diene (Minotti, 1990). Lipid peroxidation culminates in irreversible modifications of membrane structure and function. Once formed in membranes, free radical oxidized lipids can be released into extracellular fluids, presumably by the action of phospholipase A2 (Sevanian et al., 1985; Morrow et al., 1992).

The above sequence of radical species formation is initiated with the release of supernormal amounts of superoxide by doxorubicin redox cycling with the help of iron. Minotti (1990) described an additional mechanism of superoxide formation through reversal of reaction [1], although thermodynamically unfavorable, since the reduction potential for Fe (II)/Fe (III) is - 0.77 V whereas for $O_2^{\cdot}-/O_2$ is - 0.33 V. The reaction is driven by doxorubicin, an iron chelator with oxygen donor atoms and a high affinity for Fe (III), due to the phenolate and carbonyl groups present. Minotti (1990) found that the

above leads to autoxidation of Fe (II) at the expense of molecular oxygen, yielding more superoxide radicals:

$$Fe\ (II)+O_2 \rightarrow Fe\ (III)+O_2^{\cdot}- \quad \text{.....[1']}$$

Introducing competing iron chelators into biological systems challenged with doxorubicin may inhibit superoxide formation via reaction [1']. This is supported by the success of dexrazoxane, an iron chelator, which significantly reduces oxidative stress in doxorubicin treated rats (Della et al., 1999). It can be suspected that Fe (II) oxidation by the above reaction would limit its availability for the Fenton reaction. This does not appear to be the case. Minotti et al. (1995) discovered that doxorubicin is involved in a series of redox reactions liberating stored ferric iron from ferritin in human cardiomyocytes, disturbing cardiac iron balance and delivering more ferrous iron for initiation of cytotoxic Fenton reaction. Under physiologic conditions, excess Fe (II) is sequestered by ferritin, a well-shaped multi-subunit protein that is placed in the cytosolic milieu and incorporates Fe (III) by oxidizing Fe (II) with oxygen. Ferritin would subsequently release iron only when electron donors accumulate and reduce Fe (III) to Fe (II). Minotti et al. (1995) described a secondary doxorubicin metabolite, doxorubicinol, which liberates ferrous iron in a 1 (doxorubicinol) : 2 (Fe II) stoichiometric ratio. Doxorubicin is comprised of a quinone-containing tetracycline ring and a two-carbon side chain, having a carbonyl group at C-13 and a primary alcohol at C-14 (Figure 2.1). An aminosugar (daunosamine) is attached by glycosidic bond to the C-7 of the tetracycline ring (Minotti et al., 1995). *In vitro* and *in vivo* studies have shown that NADPH oxidoreductases of mitochondrial, nuclear, and microsomal membranes in human cardiomyocytes support a one-electron redox cycling of doxorubicin, and this

process is relevant for iron delocalization and cardiac damage. The side chain C-13 carbonyl group of doxorubicin is liable to a two electron reduction (Figure 2.1), yielding a secondary alcohol called doxorubicinol (DOXol). Pancytosolic enzymes that utilize NADPH as a source of reducing equivalents, classified as aldo-keto reductases or carbonyl reductases, catalyze this reaction (Minotti et al., 1990).

Doxorubicinol

Doxorubicinol (Figure 2.1) is the primary circulating metabolite in doxorubicin treated patients and laboratory animals (Cusack et al., 1993). Pharmacokinetic studies

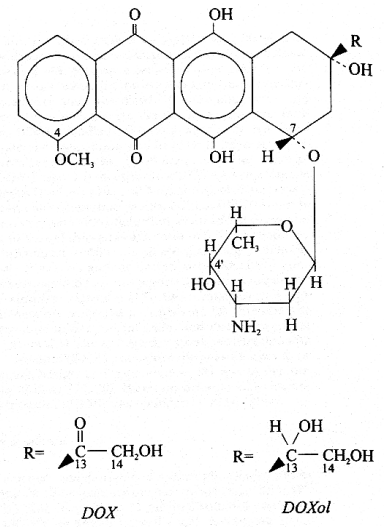

Figure 2.1 Chemical structures of doxorubicin and doxorubicinol.

in doxorubicin treated animals indicate that cardiac accumulation of doxorubicinol reflects intramyocardial drug metabolism rather than the uptake of the metabolite from the bloodstream (Mazzanti et al., 1988).

Minotti et al. (1995) used human myocardium tissue, obtained from autopsies or open heart surgery, to show that NADPH and doxorubicin supplemented cytosolic

fractions release Fe (II), and that the mobilization of Fe (II) was mediated by the drug metabolite doxorubicinol. This occurs by a nonenzymatic reduction of nonheme Fe (III). The process consists of two steps as illustrated (Figure 2.2):

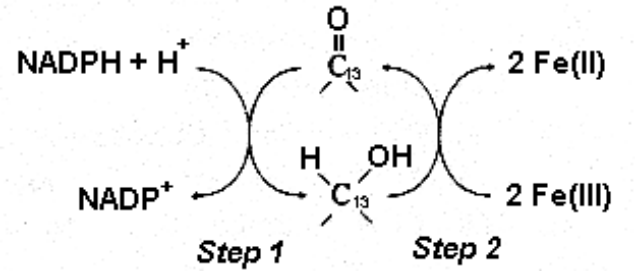

Figure 2.2 Conversion of doxorubicin to doxorubicinol (Minotti et al., 1995).

Step one consists of an enzymatic two-electron reduction of doxorubicin to doxorubicinol, whereas step two consists of a nonenzymatic two-electron mobilization of two ferrous cations. These two steps occur in human and animal cardiomyocytes, which possess the NADPH-dependent enzymatic machinery to catalyze the first step (Minotti et al., 1995). Minotti et al. (1998) discovered that iron mobilized by doxorubicinol is at the expense of the [4Fe-4S] cluster of cytoplasmic aconitase. Aconitase is a protein displaying marked homology with the mitochondrial enzyme that converts citrate to isocitrate via the intermediate *cis*-aconitase in the Krebs cycle. Under physiologic conditions, cluster disassembly abolishes aconitase activity and forms an apoprotein that binds to mRNAs, coordinately increasing the synthesis of transferrin receptor but decreasing that of ferritin. Aconitase is converted to an iron regulatory protein-1 (IRP-1) which causes iron uptake to prevail over sequestration, thereby forming a pool of free iron that is used for metabolic functions. Cluster reassembly converts IRP-1 back to aconitase, providing a regulatory mechanism to decrease free iron when it exceeds

metabolic requirements. Doxorubicinol mediates aconitase iron release and cluster disassembly, abolishing aconitase activity and irreversibly affecting the ability of the apoprotein to function as an IRP-1 or to reincorporate iron within a new Fe-S group. This damage reflects oxidative modifications of aconitase thiol residues having the dual role to coordinate cluster assembly and facilitate interactions of IRP-1 with mRNAs. The result is a disruption of iron homeostasis in human cardiomyocytes (Beinert and Kennedy, 1993). Iron chelators may have a protective effect in doxorubicin treated mammalian hearts (Minotti et al., 1998). A remarkable degree of protection is reported with dexrazoxane, a *bis*-keto-piperazinedione that hydrolyzes intracellularly and liberates a diacid diamide that chelates iron (Buss and Hasinoff, 1993).

Doxorubicin mediates heart damage through a free radical mechanism, and the role of endogenous antioxidant defenses of cardiomyocytes is critical in mitigating the damage. One such enzyme, superoxide dismutase, is produced relatively less in cardiomyocytes compared to other cell types, due to a selective decrease of the copper-requiring cytosolic enzyme (Borrello et al., 1992). The major cellular defense from free radical damage is glutathione (GSH), a tripeptide synthesized by glutathione synthetase. In its reduced form, glutathione plays an important role in the regulation and inactivation of a wide range of reactive intermediates generated intracellularly. Maintenance of an adequate intracellular pool of reduced glutathione is crucial for continued activity of several peroxidase enzymes, and for the electrophile conjugating activity of the glutathione-S-transferase enzyme family. Adequate reduced glutathione stores are maintained through the activity of glutathione reductase, which catalyzes the reduction of oxidized glutathione (GSSG) using NADPH as a source of reducing equivalents (Floche

and Gunzler, 1976). Glutathione peroxidases use glutathion to catalyze the breakdown of lipid peroxides to less toxic alcohol. Glutathion peroxidases break down hydrogen peroxide to nontoxic water and thus reduce its availability for the Fenton reaction (Mannervik, 1985). Doxorubicin causes a significant depletion of glutathione levels in cardiac myocytes. This was discovered by Paranka and Dorr (1994) using cultured heart cells from neonatal rats to quantitate the effect of doxorubicin on intracellular glutathione levels. They described a doxorubicin-mediated significant loss in oxidized and reduced forms of glutathione in the cultured heart cells.

Secondary Cytotoxic Messengers

Free radicals are short lived and thus inflict damage only in the local environment where they are produced. Lipid peroxidation results in the production of a great variety of stable, diffusable saturated and unsaturated aldehydes including malondialdehyde, 4-hydroxy alkenals, alkanals, 2-alkenal, and 2,4-alkadienals. These cytotoxic aldehydes are extremely active, and they can diffuse within or even escape from the cell and attack targets far from the initial site of the free radical event, and therefor act as secondary cytotoxic messengers. Malondialdehyde and 4-hydroxy-non-2-enal (HNE), which are α,β-unsaturated aldehydes, can be formed by peroxidation of ω-6 unsaturated fatty acids such as linoleic and arachidonic acids (Esterbauer et al., 1986). HNE exhibits a variety of cytopathological effects such as enzyme inhibition, inhibition of DNA and RNA synthesis, inhibition protein synthesis, and inhibition of heat shock proteins. It also has genotoxic and mutagenic effects as well as inhibitory effects on cell proliferation (Alary et al., 1995). Aldehydes in rat plasma and heart tissue increase significantly following *in vivo* doxorubicin treatment. Cytotoxic aldehydes produced as a consequence of

doxorubicin induced lipid peroxidation are the main culprits of the short term abnormalities, and initiate the signals leading to the chronic, long term injury, particularly with repeated exposure. The reactions initiated by the cytotoxic aldehydes result in impaired energy production, protein and DNA damage, and the induction of inflammatory reactions. This leads to increased synthesis of adhesion molecules and cytokines, infiltration of neutrophils and macrophages, and necrotic or apoptotic death of heart cells (Luo et al., 1997). Reduced glutathion contains an electron rich sulfhydryl group, which can intercept aldehydes by conjugating with them or by inhibiting their formation by free radical scavenging (Witz, 1989). Luo et al. (1997) confirmed the involvement of free radicals, and suggested that cytotoxic aldehydes play a key role in initiating the steps that lead to functional impairment of myocardium following doxorubicin administration. They also reasoned that free radical scavenging and enzymatic removal of these aldehydes might play a role in protecting the myocardium from injury.

Mitochondrial Membrane Damage

Praet and Ruysschaert (1993) established a relationship between cardiotoxicity, mitochondrial toxicity and the molecular structure of doxorubicin. They identified two sites on doxorubicin as crucial. The first is the amine in position 3' of the daunosamine, essential for the interaction with cardiolipin, which is a phospholipid specific of the inner mitochondrial membrane. The second is the quinone moiety of doxorubicin, involved in the production of free radicals. Doxorubicin binds with high affinity to cardiolipin, and is subsequently converted by NADH dehydrogenase into a semiquinone radical. In the presence of O_2, this radical generates superoxide anions and hydroxyl radicals, which

peroxidize unsaturated membrane lipids. This results in a general disturbance of the inner mitochondrial membrane structure and of its essential biological functions. Doxorubicin inhibits *in vitro* and *in vivo* the activity of complex I-III and complex IV of the mitochondrial respiratory chain, peroxidizes mitochondrial lipids, and modifies the fluidity of the mitochondrial membrane. Free radical scavengers abolish or delay these toxic effects. *In vitro* experiments using electron spin resonance techniques show increased production of hydroxyl radicals in doxorubicin perfused heart preparations, confirming drug mediated free radical production (Rajagopalan et al., 1988).

DNA Expression and Contractile Function Compromise

Doxorubicin interacts with nucleic acids and nuclear components. Doxorubicin interferes with DNA strand separation and helicase activity, and initiates DNA damage via inhibition of topoisomerase II (Gewirtz, 1999). This leads to disruption the cardiac specific program of gene expression, including mitochondrial electron transport chain components, causing ATP depletion (Jeyaseelan et al., 1997). Doxorubicin lowers mRNA levels for cardiac specific α-actin, troponin I, myosin light chain II, M isoform of creatine kinase, and mRNA expression for sarcoplasmic reticulum Ca^{2+} proteins responsible for calcium handling (Arai et al., 1998). Chronic doxorubicin treatment reduces active and rigor tension in membrane-permeabilized trabeculae isolated from right ventricular walls of rats (Bottone et al., 1998). Doxorubicin and its metabolite doxorubicinol inhibit ATPases of sarcolemma, sarcoplasmic reticulum, and mitochondrial membranes from dog or rabbit heart via a free radical mechanism (Boucek et al., 1987; Olson et al., 1988). Superoxide anion reacts with the iron-sulfur cluster associated with aconitase at a rapid rate ($k \approx 10^6\ M^{-1}\ s^{-1}$). Aconitase inactivation, in

addition to disrupting iron homeostasis, may also reduce the amount energy generated by the Krebs cycle for contractile function (Flint et al., 1993).

Endoplasmic Reticulum Damage

In rat cardiomyocytes, doxorubicin induces endoplasmic reticulum membrane damage. The endoplasmic reticulum is critical for catalyzing the desaturation and elongation of fatty acids. Doxorubicin has at least dual negative effects, the first directed against the membrane highly unsaturated fatty acids (HUFAs), and the second against the system which synthesizes HUFA from their precursors, linoleic and α-linolenic acids. Doxorubicin induces alterations in the expression of genes encoding proteins that desaturate and elongate linoleic and α-linolenic acids, causing a decline in HUFA synthesis. The second effect stems from products originating from the peroxidation of microsomal lipids (i.e. secondary cytotoxic messengers), which have been demonstrated to damage the microsomal proteins catalyzing HUFA synthesis (Bordoni et al., 1999).

Most of the work on doxorubicin induced cardiotoxicity implicates a free radical mechanism, with overproduction of superoxide anion and subsequent production of iron-catalyzed hydroxyl radical as the initial stage. Endogenous antioxidant exhaustion and secondary cytotoxic messenger release further lead to doxorubicin induced cardiotoxicity and heart failure. In developing cardioprotective strategies for doxorubicin toxicity, many compounds offer possible prophylactic action. The following discussion reports success with the use of various exogenous antioxidants, endogenous antioxidant boosters, and iron chelators.

Cardioprotectant Research

Iron Chelators

Dexrazoxane, also referred to as ICRF-187 or ADR 529, is a *bis*-keto-piperazinedione that hydrolyzes intracellularly and liberates a diacid diamide, which in turn chelates iron (Buss and Hasinoff, 1993). Speyer et al. (1988) demonstrated significant cardioprotection with the use of dexrazoxane in breast cancer patients treated with doxorubicin. Della et al. (1999) evaluated the safety and cardioprotective activity of dexrazoxane in weanling rats given doxorubicin. They demonstrated significantly reduced toxicity in the heart, liver, and kidneys of rats in the group treated with both drugs for seven weeks, versus the group treated with doxorubicin alone. Another mouse group was given both doxorubicin and the iron chelator to demonstrate a continual cardioprotection after four months from cessation of treatment. Dexrazoxane does not interfere with the anti-neoplastic activity of doxorubicin in children with acute lymphoid leukemia (ALL), which suggests that an iron chelator *per se* does not suppress doxorubicin's tumorcidal action (Schuler et al., 1997).

Endogenous Antioxidant Mimics

Eugene et al. (1999) developed a cell culture model for doxorubicin induced myocardial injury using primary adult rat cardiomyocytes exposed to clinical concentrations of the drug. For potential cardioprotectants, they used a cell permeable superoxide dismutase mimic (MnTBAP) and ebselen, a cell permeable glutathione peroxidase mimic. To measure the amount of doxorubicin induced damage, they measured release of sarcosolic enzyme lactate dehydrogenase released from the cultured cardiomyocytes into a serum free medium. They also assessed aconitase activity in cell

cultures. Doxorubicin caused a dose dependent release of lactate dehydrogenase from the cultured cardiomyocytes. The release of lactate dehydrogenase was prevented with MnTBAP, although unaffected by cell impermeable superoxide dismutase. Ebselen enhanced the protection of cardiomyocytes afforded by MnTBAP. Doxorubicin increased the amount of intracellular superoxide in cardiomyocytes, which was significantly reduced with MnTBAP addition. MnTBAP partially reversed the inactivation of aconitase induced by doxorubicin, and ebselen further amplified this protective effect. Biochemical data was supported with morphological examination of cell damage (Figure 2.3).

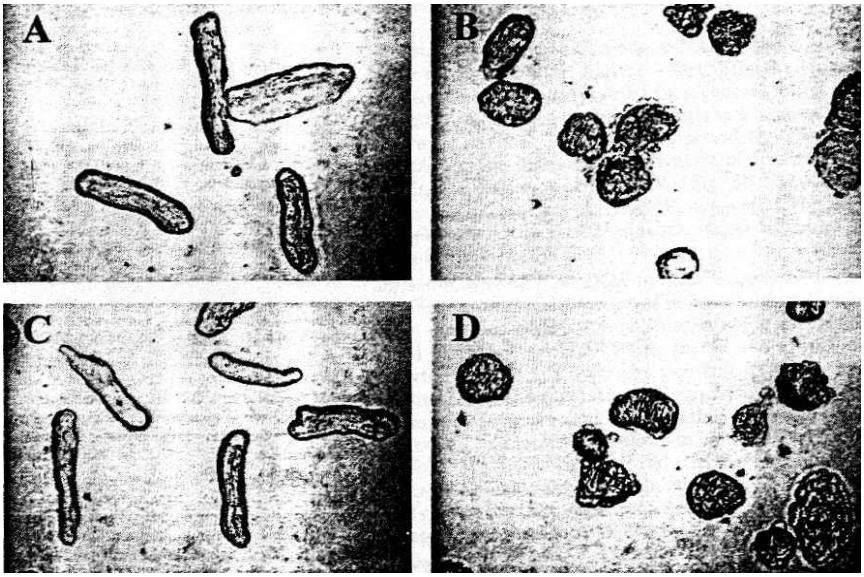

Figure 2.3 Phase contrast images of mouse cardiomyocytes at 100 X magnification (Eugene et al., 1999).

After 48 hours in culture, most of the control cells retained their rod-like shape with clearly visible cross striation (Figure. 2.3A). After doxorubicin treatment, all cells were rounded with subsarcolemmal blebs and showed a lack of intact myofibrillar organization (Figure. 2.3B; Figure 2.3D). MnTBAP preserved the myofibrillar structure

and prevented doxorubicin induced structural damage such as cell rounding and subsarcolemmal bleb formation (Figure 2.3C). The study demonstrated that the superoxide dismutase mimic MnTBAP prevents doxorubicin induced damage to cardiomyocytes and that the glutathione peroxidase mimic ebselen synergistically enhanced the cardioprotection of MnTBAP (Eugene et al., 1999). This is in agreement with an earlier report showing that overproduction of Mn-SOD by transgenic mice completely protects these animals from doxorubicin induced cardiotoxicity (Yen et al., 1996).

Enhancing Cardiac Glutathion Synthesis

Glutamine becomes rate limiting for the synthesis of glutathione during stress such as chemotherapy induced oxidative injury. Glutamine supplementation leads to upregulation of cardiac glutathione synthesis (Welborne, 1979). Oral glutamine supplementation increases blood and cardiac tissue glutamine and glutathione levels, despite doxorubicin co-treatment in rats (Cao et al., 1999).

Flavonoids

α G-Rutin and luteolin have phenolic hydroxy groups that can trap free radicals. These compounds suppress doxorubicin induced lipid peroxidation in mouse heart and liver. Flavonoids restore cardiac glutathione levels in mice co-treated with doxorubicin. *In vivo* studies showed luteolin and α-tocopherol to be more effective in suppressing lipid oxidation induced by doxorubicin than α G-Rutin, and *in vitro* studies showed the opposite. The difference in the results between *in vitro* and *in vivo* results is caused by the differences in the solubility of these molecules. α G-Rutin is hydrophilic and

immediately metabolized and excreted in the body, whereas luteolin and α-tocopherol are lipophilic, slowly metabolized and stored in the body. Intraperitoneal and oral administration of the flavonoids yielded similar results in mice (Sadzuka et al., 1997).

Thymoquinone

Al-Shabanah et al. (1998) successfully used thymoquinone, a product derived from the oil of *Nigella sativa* seeds, as a cardioprotectant in doxorubicin treated mice. Thymoquinone is a membrane lipid antioxidant and a strong scavenger of superoxide radicals. They collected and analyzed biochemical and histological data from control, doxorubicin treated mice, and thymoquinone supplemented mice on doxorubicin. They observed significant protection from cardiac damage in the latter group, and suggested a free radical scavenging activity as a mechanism of protection. They also conducted a survival study in mice bearing Ehrlich Ascites Carcinoma tumor to assess thymoquinone's effect on the antitumor activity of doxorubicin. Mice on combined treatment showed a fifty percent survival at sixty days after drug treatment compared to a thirty percent survival for mice on doxorubicin alone.

Carvedilol

Carvedilol is a β-blocker with α_1-blocking vasodilatory properties and several other potentially beneficial effects, including an antioxidant effect (Feuerstein and Ruffolo, 1998). Matsui et al. (1999) measured cardiac performance and myocardial lipid peroxidation in rats treated with doxorubicin, with or without carvedilol administration. They compared the effects of carvedilol with those of atenolol, a β-blocker without antioxidant properties. Carvedilol, administered orally or intraperitoneally, reduced lipid

peroxidation and restored cardiac function to normal levels in rats treated with doxorubicin. It is unlikely that the cardiotoxicity was prevented by β-receptor blockade *per se*, because the absence of such protection with atenolol treatment in their study. This supports the antioxidant action of carvedilol as the source of protection.

Melatonin

Melatonin, a pineal hormone, is a powerful antioxidant, which suggests its usefulness in treating oxygen radical pathophysiology (Reiter et al., 1997). Morishima et al. (1998) studied the potential cardioprotection offered by melatonin in doxorubicin treated rats. Mortality occurred exclusively in rats treated with doxorubicin alone. When compared to rats in the control group, surviving rats in the doxorubicin alone group showed decreased heart to body weight ratios, increased lipid peroxidation, myocardial lesions, accumulation of ascites, and functional compromise of the heart muscle. Rats in the group given both doxorubicin and melatonin displayed biochemical, histological, and functional parameters similar to those in the control group. Melatonin has amphipathic properties; it is both lipophilic and hydrophilic, so it can be carried by the plasma and protect anywhere it is needed in the body (Shida et al., 1994). By attenuating lipid and aqueous peroxidation, melatonin is highly effective in protecting rats from doxorubicin induced cardiotoxicity (Morishima et al. 1998).

α-Lipoic Acid

Structure

α-Lipoic acid (LA), also known as lipoate, thioctic acid, or 6,8-dithiooctanoic acid is an eight carbon disulfur compound (Figure 2.4). In the 1950s, lipoate was

identified as an essential cofactor in oxidative metabolism. Biologically, lipoate exists as lipoamide in at least five proteins where it is covalently linked to a lysyl residue. Four of these proteins are found in α-keto acid-dehydrogenase complexes, which consists of the pyruvate dehydrogenase complex, α-ketoglutarate dehydrogenase complex, and the branched chain α-keto acid dehydrogenase complex. The fifth lipoamide moiety is part of the glycine cleavage system, which catalyzes the reversible oxidation of glycine (Fujiwara et al., 1991).

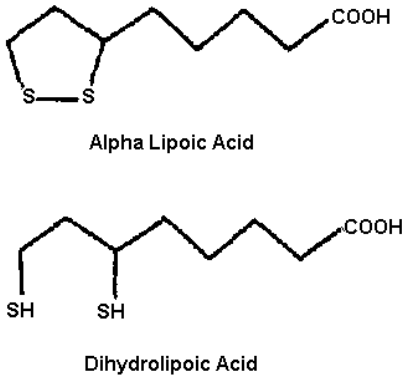

Figure 2.4 Structures of α-lipoic acid and dihydrolipoic acid.

α-Lipoic acid, in its native form, contains a disulfide bond. Reduction of this disulfide results in the conversion of α-lipoic acid to the corresponding vicinal dithiol, dihydrolipoate (DHLA), also known dihydrolipoic acid, or 6,8-dimercaptooctanoic acid. Dihydrolipoate is the more potent reductant and reacts with free radical species not acted upon by the oxidized form, α-lipoate. Dihydrolipoate is also the form that shares its reducing power with other antioxidants (Packer and Tritschler, 1996).

Reduction of α-Lipoic Acid to Dihydrolipoic Acid

Cellular reducing equivalents such as NADH or NADPH produced as a result of cellular metabolism, serve as cofactors for enzymes such as reductases or dehydrogenases in bioreduction processes. A distinct property of intercellular α-lipoic acid is that it is a metabolic antioxidant, because enzymatic systems in mammalian cells treat it as a substrate for bioreduction. The characteristics of uptake and distribution of α-lipoic acid in mammalian cells are well examined. Orally supplied α-lipoic acid is rapidly absorbed in the gastrointestinal tract, but is subject to some presystemic elimination in the liver, releasing about a third of it into the blood stream. Erythrocytes possess a bidirectional transport system for both α-lipoic acid and dihydrolipoic acid. Such transport systems are ubiquitous in mammals such as rats and humans. Supplemented α-lipoic acid is transported to cells, including those in the heart, and reduced to dihydrolipoic acid at the expense of cellular reducing equivalents (Constantinescu et al., 1995).

The reduction mechanism for α-lipoic acid depends on its stereochemistry. α-lipoic acid exists as either R- or S-enantiomers, and the R-enantiomer is the form naturally existing in cells. The mitochondrial E3 enzyme, dihydrolipoyl dehydrogenase, preferentially reduces the R-enantiomer of α-lipoic acid to dihydrolipoic acid at the expense of NADH (Haramaki et al., 1997). The S-enantiomer is a substrate for NADPH-dependent glutathione reductase in the cytosol. The rate of reduction to dihydrolipoic acid is much slower than that of glutathione disulfide, the natural substrate. Thioredoxin reductases from calf thymus and liver, human placenta, and rat liver efficiently reduce α-lipoic acid (Arner et al., 1996).

The pathway of α-lipoic acid reduction varies in different tissues, since it is highly dependent on mitochondrial content. NADH contributes about ninety percent of α-lipoic acid reduction in the heart, sixty three percent in kidney, and fifty percent in liver. Erythrocytes, which contain no mitochondria, reduce α-lipoic acid almost exclusively by NADPH-dependent cystolic enzymes (Packer and Tritschler, 1996; Haramaki et al., 1997).

α-Lipoic Acid: The Amphipathic and Therapeutic Antioxidant

Thiols are widely regarded as possessing antioxidant and cardioprotective effects by virtue of their reducing properties. Dihydrolipoic acid is a potent sulfhydryl reductant with a DHLA/LA redox potential equal to - 0.32 V, compared with - 0.24 V for reduced glutathion (GSH) / oxidized glutathione (GSSG) (Searls and Sanadi, 1960). In the United States, dihydrolipoic acid, α-lipoic acid, and derivatives have been intensively investigated as antioxidants. In Germany, α-lipoic acid has long been used as a therapeutic agent in a variety of diseases involving enhanced free radical peroxidation of lipid membrane phospholipids, including liver cirrhosis and diabetic neuropathies (Ziegler et al., 1995; Altenkirch et al., 1990). Packer et al. (1995) demonstrated that dihydrolipoic acid directly scavenges superoxide radicals, singlet oxygen, hydroxyl radicals, peroxyl radicals, and hypochlorous acid while being itself oxidized to α-lipoic acid. Kagan et al. (1992) found that dihydrolipoic acid is an efficient direct scavenger of both water-soluble and lipid soluble peroxyl radicals. This scavenging can occur in the cytosol or the hydrophobic domains of membrane bilayers with an estimated stoichiometric ratio of 1.5 mol of peroxyl per mol of dihydrolipoic acid. In contrast to many other antioxidants, dihydrolipoate functions as a universal free radical scavenger

that neutralizes peroxyl radicals throughout the cell. Oxidized α-lipoic acid does not offer the same protection, and mitochondrial reduction of α-lipoic acid to dihydrolipoic acid may constitute the source of free radical chain terminating activity (Kagan et al., 1992).

Matsugo et al. (1995) used the organic hydroperoxide, NP-III, which produces hydroxyl radicals on illumination by UVA light (>320 nm). Apolipoprotein (apo-B) of human low density lipoprotein (LDL) and bovine serum albumin (BSA) were irradiated with UVA in the presence of NP-III and dihydrolipoic acid. Oxidation of BSA and apo-B of LDL was completely inhibited by dihydrolipoic acid. The results showed that dihydrolipoic acid is a direct and efficient hydroxyl radical scavenger. Chevion et al. (1997) showed that α-lipoic acid can neutralize the peroxyl radical ($ROO^{\cdot}$) in vitro.

Metal Chelating by α-Lipoic Acid

An alternative, non-radical scavenging mechanism by which α-lipoic acid acts as an antioxidant is via its metal chelating capacity. α-Lipoic acid inhibits iron dependent oxidative reactions by rendering it redox inactive through ligation to its sulfur residues (Scott et al., 1994). Additionally, Bonomi and Pagani (1991) described an iron-dihydrolipoate binuclear (FeDHL) complex, which is stable over a wide physiological pH range:

$$[Fe_2(\text{dihydrolipoate})_3]^{3-} \Leftrightarrow 2\ Fe^{3+} + 3\ \text{dihydrolipoate}^{3-}$$

Iron binding, by either α-lipoic acid or dihydrolipoic acid, may reduce the amount of hydroxyl radical production via iron mediated Fenton reactions. Copper, also a free radical catalyst, is susceptible to chelation by α-lipoic acid's thiol group, as with iron.

By doing so, α-lipoic acid inhibits Cu^{2+} catalyzed liposomal peroxidation, ascorbic acid oxidation, and ascorbate catalyzed peroxide formation in erythrocytes. α-Lipoic acid needs not to be reduced in order for it to carry out such protective function (Ou et al., 1995).

Regenerating Other Cellular Antioxidants

Coenzyme Q_{10}. Ubiquinone (UQ), also known as coenzyme Q_{10}, is a natural constituent involved in mitochondrial energy metabolism. Its bioenergetic activities require redox cycling, the same as with α-lipoic acid. In the case of α-lipoic acid, redox cycling leads to dihydrolipoic acid which is involved in the Krebs cycle, while ubiquinone recycles through semi- and divalently reduced ubiquinones in the respiratory chain. Ubiquinol (UQH_2), the reduced form of ubiquinone, interferes with lipid peroxidation membranes by undergoing two consecutive oxidation steps to ubiquinone. The resulting product has no protective power and is dependent on a recycling system that transforms it back to its antioxidant form.

Dihydrolipoate recycles ubiquinone to ubiquinol by a two-electron transfer step, being itself oxidized to α-lipoic acid that is readily reduced back by NADPH or NADH to dihydrolipoate (Nohl and Gille, 1998). Kazlov et al. (1999) showed that dihydrolipoic acid maintains ubiquinol in the antioxidant form in rat liver mitochondria by a two-electron reduction of ubiquinone (UQ), and by a one-electron reduction of ubisemiquinone ($UQH^{\cdot}$ / $UQ^{\cdot -}$). The latter is widely regarded as the electron transport chain component responsible for superoxide production (Cadenas et al., 1977). Data from both studies supports a synergistic effect of α-lipoic acid in combination with coenzyme Q_{10} in attenuating free radical production.

Vitamins C and E. Kagan et al. (1992) found that dihydrolipoic acid directly reduces ascorbyl radicals generated by free radical oxidation of ascorbate, also known as vitamin C. They described an ascorbate-mediated dihydrolipoic acid-dependent reduction of vitamin E chromanoxyl radicals, which means that dihydrolipoic acid indirectly recycles vitamin E using vitamin C. To study this interaction, they generated chromanoxyl radicals from vitamin E using peroxyl radicals:

$$ChR\text{-}OH + LOO^{\cdot} \rightarrow ChR\text{-}O^{\cdot} + LOOH$$

They also exposed vitamin E to ultraviolet radiation:

$$ChR\text{-}OH + h\nu \rightarrow ChR\text{-}O^{\cdot} + e^{-} + H^{+}$$

The amounts of chromanoxyl and ascorbyl radicals were measured *in vitro* using electron spin resonance spectrophotometry (ESRS). Introduction of ascorbate temporarily reduced the ESRS signal generated by chromanoxyl radicals, indicating some vitamin E recycling, while dihydrolipoic acid alone or α-lipoic acid alone did not have such effect.

Dihydrolipoic acid reduced the ESRS signal generated by ascorbyl radicals, and this effect was greatly enhanced with the addition of NADPH or NADH. When chromanoxyl radicals were added to a microsomal or liposomal fraction containing dihydrolipoic acid, ascorbate, and NADPH/NADH, this lead to a complete and lasting elimination of chromanoxyl radicals in favor of vitamin E regeneration. Dihydrolipoic acid maintains a high steady-state concentration of ascorbate by reducing it from semidehydroascorbate and dehydroascorbate, thereby enhancing vitamin E recycling. Dihydrolipoic acid bolsters antioxidant systems by interacting with other redox couples to synergistically enhance their ability to recycle vitamin E (Figure 2.5).

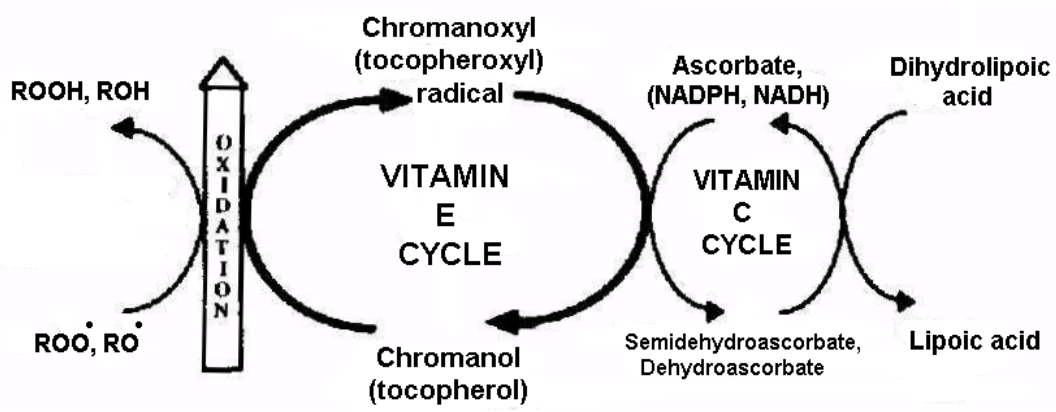

Figure 2.5 Scheme explaining the interaction between α-lipoic acid and vitamin C in regenerating vitamin E (Kagan et al., 1992).

Vitamin E and glutathione. Bast and Haenen (1990) investigated dihydrolipoate's involvement in an earlier discovered vitamin E regeneration mechanism. Vitamin E is recovered from chromanoxyl radicals by membrane bound vitamin E free radical reductase, and thus ensures that cellular antioxidant capacity is not overwhelmed easily by free radical attack. Reduced glutathione functions as a cofactor for this important enzyme by donating the reducing equivalents that drive this process. Since reduced glutathione is a thiol compound, they investigated whether reduced α-lipoic acid, another thiol compound, can substitute for reduced glutathione as a cofactor for vitamin E reductase. They showed that this was not the case. Oxidized glutathione also fails to drive the process, but the addition of dihydrolipoic acid leads to a pronounced reductase activity. Replenishing cellular vitamin E by combining oxidized glutathione and reduced α-lipoic acid proceeds via formation of reduced glutathion, the essential cofactor for chromanoxyl reductase (Figure 2.6). Reduction of oxidized glutathion by dihydrolipoate is thermodynamically feasible given the reduction potential for both redox couples: - 0.32 V for DHLA / LA, *vs*. - 0.24 V for GSH / GSSG (Searls and Sanadi, 1960). The above

studies show that α-lipoic acid, upon its reduction, strengthens the antioxidant network by regenerating key cellular free radical defense components, including reduced glutathione, vitamin E, and coenzyme Q_{10}.

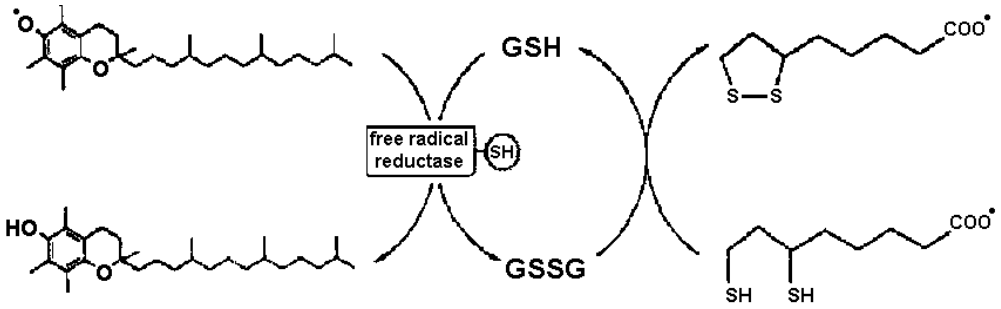

Figure 2.6 Interplay between lipoic acid, glutathione, and free radical reductase in the regeneration of vitamin E (Bast and Haenen, 1990).

Increasing de novo glutathione synthesis. α-Lipoic acid induces a substantial increase in cellular reduced glutathione synthesis in many cells, including human lymphocytes and erythrocytes. Glutathione synthesis requires cellular cysteine uptake. Cellular reduction of α-lipoic acid to dihydrolipoic acid allows it to leave the cell and reduce plasma cystine to cysteine. This is thermodynamically feasible, since the cysteine/cystine redox potential is - 0.22 V. Cysteine is readily taken up by the cellular neutral amino acid transport system (ACS), a well expressed uptake pathway in human cells (Figure 2.7). Intercellular cysteine is critical, and often rate limiting, for the function of γ-glutamylcysteine synthetase, a key enzyme in glutathione synthesis (Han et al., 1997).

Synthesized glutathione is continuously eliminated from most cells by canalicular GSH transporters, the same fate awaits glutathione after oxidization following free

radical quenching. Once transported outside the cell, both forms of glutathione are rapidly cleared from circulation by renal illumination.

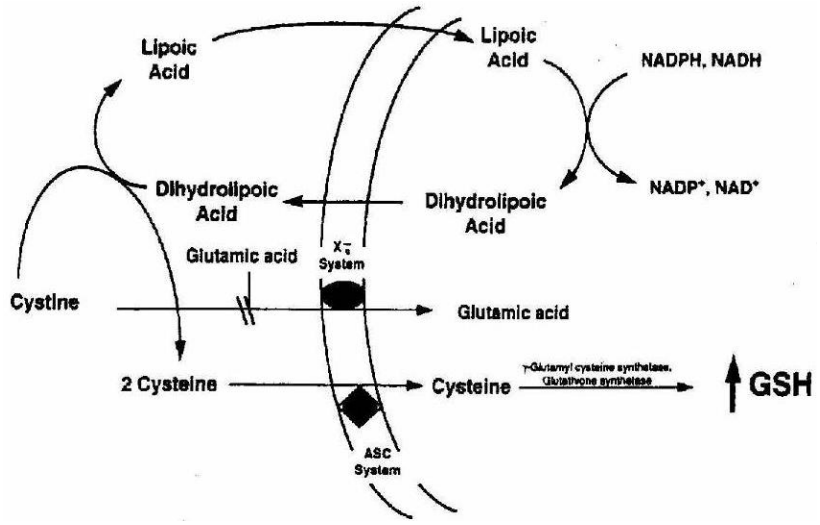

Figure 2.7 Schematic illustration of *de novo* glutathione synthesis mediated by α-lipoic acid (Han et al., 1997).

Glutathione transports work bidirectionally in principle, but net uptake is negligible due to the concentration gradient and rapid extracellular elimination. Maintenance of cellular glutathione therefore depends on its *de novo* synthesis in most tissues. When handling natural levels of free radicals, such as superoxide released from the electron transport chain, cells can maintain glutathione levels adequately. In cases of abnormally elevated oxidative stress such as doxorubicin treatment, glutathione is used up and eliminated much more rapidly. Synthesizing glutathione also becomes more difficult, since uptake of cysteine is inhibited due to its oxidation by free radical species (Bannai, 1984). Han et al. (1997) showed that α-lipoic acid supplementation elevates *de novo* glutathione synthesis by reducing extracellular cystine to cysteine, allowing for its cellular uptake and utilization.

Effectiveness of α-Lipoic Acid in Treating Oxidative Stress

Oxidative stress and age associated metabolic decline. Mitochondrial dysfunction contributes to the increased oxidative stress and loss of function accompanying aging (Sastre et al., 1996; Shigenaga et., 1994). Oxygen is used less efficiently in mitochondria from aged tissue, leading to impaired ATP synthesis and increased oxidant production. The high flux of oxidants damages the mitochondria and cellular biomolecules at an increased rate, especially since endogenous antioxidant defenses also decline with age (Sanz et al., 1997; Sohal and Weindruch, 1996). This leads to progressive mitochondrial decay, inadequate energy production, and the general metabolic decline evident with aging. Age associated functional impairment, and doxorubicin induced cardiac failure, parallel each other to a certain extent, since increased oxidative stress is strongly associated with both (Hagen et al., 1999).

Hagen et al. (1999) investigated the effects of α-lipoic acid supplementation on cellular and general metabolic activity, hepatocellular antioxidant status, oxidant production, and oxidative damage in rats. They used young and old rats on a diet with or without α-lipoic acid (0.5% w/w) for two weeks. α-Lipoic acid supplemented old rats showed a marked reversal in age associated oxygen consumption decline. The mitochondrial membrane potential in old rats increased by fifty percent with α-lipoic acid supplementation, although it was still lower when compared to young untreated rats. Physiological data showed a doubling of ambulatory activity in supplemented old rats, which was not appreciably lower than the activity observed in untreated young rats. Levels of glutathione and ascorbate were substantially depressed in old untreated rats compared to young rats, but α-lipoic acid supplementation restored both antioxidant

levels these rats. To verify that the above findings translated to reduced oxidative stress, malondialdehyde levels, a lipid peroxidation indicator, was measured in all rat groups. Malondialdehyde formation was suppressed in old rats treated with α-lipoic acid, compared the unsupplemented old rat group. Administration of α-lipoic acid stimulates insulin-dependent and independent glucose uptake into cells and enhances nonoxidative and oxidative glucose metabolism (Streeper et al., 1997). α-Lipoic acid increases ATP synthase activity, maintains critical thiol groups in a reduced state, and allows mitochondrial protein carriers to function more effectively, all which may lead to enhanced metabolism (Zimmer el al., 1991).

Cardiac ischemia and reperfusion. A reliable method for inducing oxidative damage in the heart, for evaluation of antioxidant protection, is to reperfuse it after a brief period of ischemia. Schonheit et al. (1995) evaluated a possible interference of α-lipoic acid with cardiac ischemia and reperfusion injury at the level of the intact organ and at the subcellular level of mitochondria. In order to follow the effect of α-lipoic acid, the isolated perfused organ was subjected to total global ischemia and reperfusion in the presence and absence of different concentrations of α-lipoic acid. Treatment with 5 μM of α-lipoic acid improved the recovery of hemodynamic parameters, although electrophysiological parameters were not influenced. Application of 10 μM of α-lipoic acid to rat hearts further improved the recovery of hemodynamic functions and shortened the duration of severe rhythm disturbances in comparison to reperfusion of control hearts. On a molecular level, dihydrolipoic acid reduced the formation of mitochondrial superoxide radicals. Dihydrolipoic acid controls mitochondrial superoxide formation

indirectly by regulating redox-cycling ubiquinone. Suppressing this mitochondrial superoxide generator mitigates postischemic oxidative stress, and in turn reduces damage to hemodynamic and electrophysiological heart functions (Schonheit et al., 1995).

Diabetic peripheral and cardiac neuropathies. Diabetic peripheral neuropathy is complication of diabetes characterized by pain, burning, paresthesia, and numbness of the extremities. Blood sugar control is generally accepted as the primary approach for prevention of diabetic peripheral neuropathy. Retarding advanced diabetic nerve dysfunction requires several months or even years of strict glycemic control. In symptomatic diabetic neuropathy, pharmacological treatment of painful neuropathic symptoms is frequently required to maintain the patient's quality of life (Kennedy et al., 1990). Cardiovascular autonomic neuropathy is an additional serious complication of diabetes with a poor prognosis associated with an irregular heart rhythm (Ziegler, 1994). A growing body of evidence suggests that oxidative stress, due to enhanced free radical formation or defects in antioxidant defense, is implicated in diabetic neurodegenerative disorders (Baynes., 1991). Elevated levels of plasma hydroperoxides in conjunction with lower vitamin E levels have been demonstrated in patients with diabetic neuropathy (Yoshida et al., 1995; Nourooz-Zadeh et al., 1995). In experimental diabetic neuropathy, oxygen free radical activity in the sciatic nerve is increased (Cameron and Cotter, 1994).

Ziegler and Gries (1997) investigated the effects of α-lipoic acid on diabetic neuropathy in two multi center, randomized, double blind, placebo controlled trials. In the first trial, patients were randomly assigned to treatments with intravenous infusions of α-lipoic at doses up to 1,200 mg or placebo over a three week period. The total symptom score (pain, burning, paresthesia, and numbness) in the feet decreased considerably from

baseline to day 19 using 1,200 mg or 600 mg per day of oral α-lipoic acid *vs*. placebo group. Cardiac autonomic dysfunction was reduced in the second trial using an oral α-lipoic acid dose of 800 mg per day for four months *vs*. the placebo group.

Doxorubicin toxicity in rats. Many published studies have demonstrated doxorubicin toxicity protection with α-lipoic acid in rats. Malarkodi et al. (2004) studied the oxidoreductive status of red blood cells in rats treated with doxorubicin, with or without α-lipoic acid treatment. The study showed a statistically significant protective effect based on hematological indices, specifically hemoglobin and hematocrit levels. The study showed similar results in regards to lipid peroxidation levels, as well as measured levels of enzymatic (e.g. superoxide dismutase) and non-enzymatic (e.g. vitamin E) mediators of free radical protection.

Prahalathan et al. (2006) studied the toxic effect of doxorubicin on rat bone marrow cells, including DNA strand damage, chormosomal aberrations, and apoptosis frequency. α-Lipoic acid showed a protective effect in all studied indices in that study, with α-lipoic acid and doxorubicin co-treated rats showing bone marrow status similar to that of control. Kidney tissue is exquisitely sensitive to doxorubicin in rats. The molecular mechanism of doxorubicin kidney toxicity in rats has not been elucidated fully, but DNA breakage and oxidative damage cascades are highly suspected. Markers of renal cell damage and enzymatic antioxidants in doxorubicin treated rats, with and without α-lipoic acid pretreatment were measured. The results showed a significant mitigation of doxorubicin induced nephrotoxicity upon pretreatment with α-lipoic acid (Malarkodi et al., 2003).

In addition to biochemical markers of cell damage, transmission electron microscopy (TEM) has been employed by Prahalathan et al. (2006) to study the protective effect of α-lipoic acid against doxorubicin induced testicular toxicity in rats. TEM showed that germ cells from doxorubicin treated rats showed the following pathological changes: abnormal vesicles, chromatin margination along the nuclear membrane, scattered electron dense material, and mitochondrial distention with loss of cristae. Control rats and rats treated α-lipoic acid, with or without doxorubicin treatment, showed none of the TEM abnormalities exhibited by germ cells of rats treated with doxorubicin alone. The study used cellular thiol levels as an index of oxidoreductive status of cells, as well as mRNA levels for glutathione peroxidase. α-Lipoic acid treatment prevented thiol and mRNA depletions induced by doxorubicin in rats.

α-Lipoic acid and doxorubicin in rat cardiotoxicity studies. To date, two published studies have examined the effect of α-lipoic acid in doxorubicin induced cardiotoxicity, and both studies utilized biochemical markers. Balachandar et al. (2003) studied three sets of biomarkers in male Wistar rats treated with doxorubicin, with or without α-lipoic acid. The first set was lipid peroxidation markers, the second set was myocyte lytic markers (i.e. creatinephosphokinase and lactate dehydrogenase), and the third set of markers measured enzymatic and non-enzymatic antioxidant levels in heart cells. The results of that study demonstrated that α-lipoic acid offered significant cardioprotection from doxorubicin in the rat heart, and support the results of a similar study done previously by Al-Majed et al. (2002).

Safety of α-Lipoic Acid. No significant adverse events occurred in a trial published by Ziegler and Gries (1997), which emphasized the safety and effectiveness of α-lipoic acid in treating human disorders associated with enhanced oxidative stress. Hagen et al. (1999) reported no adverse side effects in rats from α-lipoic acid administration, although they did note slight weigh loss in these rats, which they attributed to enhanced metabolism.

α-Lipoic acid and the antitumor activity of doxorubicin. Interaction between α-lipoic acid and doxorubicin in rats, as well as the effects of the combination on tumor cells warrant investigation. This is necessary if α-lipoic acid is to be considered for eventual clinical trials. α-Lipoic acid may be a modulator of tumor growth, and its interaction with doxorubicin has been examined in a study by Dovinova et al. (1999). They used both compounds on L1210 mouse leukemia cells, and showed that α-lipoic acid acts as a growth factor at low concentrations (1 μM), and thus antagonizes the antiproliferation effects of doxorubicin *in vitro*. At high concentrations (100 μM), α-lipoic acid acts as an antineoplastic agent. This paradoxical antineoplastic activity attributed to α-lipoic acid, along with the mechanisms responsible, await further investigation. When using a (16 mg/kg) α-lipoic acid dose along with a high dose of doxorubicin (5 mg/kg) in leukemic mice, they also discovered synergistic tumorcidal activity; the combined dose led to a super-additive survival effect on the leukemic mice in the study.

Proposed Experiment

To administer α-lipoic acid to rats *in vivo* in order to determine if it offers cardioprotective properties to rats treated with cardiotoxic doses of doxorubicin.

Hypothesis

Administration of α-lipoic acid will offer cardioprotection in rats given cardiotoxic doses of doxorubicin. Cardioprotection parameters include mortality, cardiac function status indicated by ascites formation, cardiac mass measurements, histological changes as seen with light microscopy, and ultrastructural changes as seen in transmission electron microscopy.

CHAPTER THREE

Materials and Methods

Animals

Forty eight albino female rats (*Rattus rattus,* Harlan Sprague-Dawley, Houston, Texas) at six weeks of age were housed in the animal research facility at Baylor University. Animals were maintained in a temperature-controlled environment between 20^{o}C and 22^{o}C, under a 12-hour light and 12-hour dark photoperiod, in 46 cm x 20 cm x 25 cm suspended wire cages. Each cage housed two rats. Rats were allowed to acclimate to the research facility for one week before the four week injection period began. Rats were weighed on day one of the experiment, and once weekly thereafter, for a total of eight weighing periods.

Food

Rats were fed a normal diet (Rat Diet #5012, PMI Feeds Inc., St. Louis, Missouri) with food and water provided *ad libitum*.

Groups

Rats receiving various injection regimens were divided into four groups, with twelve rats assigned per group (Table 3.1).

Table 3.1 Rat Groups. Forty eight rats were divided into four groups (N=12) with varying injection regimens.

Group No.	Group Name	N	Test Compound
1	CON	12	(none)
2	ALA	12	α-Lipoic Acid
3	DOX	12	Doxorubicin
4	DOX+ALA	12	(Both)

Chemical Preparation

Doxorubicin Vehicle

Doxorubicin, in the concentration specified under *Dose Schedule*, was dissolved in 0.5 ml of five percent dextrose solution and administered intraperitoneally (Matsui et al., 1999).

α-Lipoic Acid Vehicle

α-Lipoic acid, in the concentration specified under *Dose Schedule*, was dissolved in 0.5 ml of 0.02 percent NaOH and administered intraperitoneally (Anuradha and Varalakshmi, 1999).

Dose Schedule

Rats in the α-lipoic acid (ALA) group were given four intraperitoneal injections of 16 mg per kg α-lipoic acid (Sigma Chemical Co., St. Louis, MO) for four weeks beginning at seven weeks of age. Rats in the doxorubicin (DOX) group were given three intraperitoneal injections of 5 mg per kg doxorubicin (Sigma Chemical Co., St. Louis, MO) over a three week period starting at eight weeks of age. Rats in the ALA group received doxorubicin vehicle according to the same regimen as the DOX group. Rats in

the DOX group received ALA vehicle according to the same regimen as the ALA group. Rats in the DOX+ALA group received three intraperitoneal injections of 5 mg per kg doxorubicin over a three week period starting at eight weeks of age, and four intraperitoneal injections of 16 mg per kg α-lipoic acid for four weeks beginning at seven weeks of age. Animals in the control group received doxorubicin vehicle and α-lipoic acid vehicle intraperitoneally according to the same regimen as the DOX+ALA group. After administrating all injections, rats were observed and then sacrificed at fourteen weeks of age.

Survival Study

All animals were observed daily for the entire duration of protocol. The general condition of each animal and deaths in each group were recorded daily. The observation period of the survival study began at seven weeks of age until the study ended at fourteen weeks of age. The data from these observations were analyzed as explained in *Statistical Analysis.*

Sacrifice and Heart Organ Collection

The rats were sacrificed by decapitation using a guillotine. The thoracic cavity was opened by midline incision, and the hearts excised. Hearts were immediately washed with ice cold 0.1 M sodium phosphate buffer (pH 7.4) and placed on blotting paper for a few seconds to absorb excess washing solution.

Gross Data Collection

Body Weight

Just before sacrifice, each animal was weighed on a digital scale. The recorded data was analyzed as indicated in *Statistical Analysis*.

Heart Weight

Immediately following sacrifice, and prior to histopathological examinations, hearts were removed, washed with buffer, and dried on blotting paper as explained previously. All hearts were then weighed on a digital scale. The recorded data was analyzed as indicated in *Statistical Analysis*.

Heart Weight to Body Weight Ratios

The pre-sacrifice body weight for each animal (B) and the post-sacrifice heart weight from the same animal (H) were used to compute the heart weight to body weight ratio (H/B) for each animal. The resulting data were analyzed as indicated in *Statistical Analysis*.

Specimen Preparation

Hearts from each group were washed in cold 0.1 M sodium phosphate buffer (pH 7.4). Samples were taken from the free left ventricular wall, between the mid region and the apex, and washed in 0.1 M sodium phosphate buffer. The samples were then fixed with two percent glutaraldehyde and two and a half percent formaldehyde in 0.1 M sodium phosphate buffer. The samples were then washed in 0.1 M sodium phosphate buffer and post-fixed with one percent osmium tetroxide in 0.1 M sodium phosphate buffer. The samples were then dehydrated in graded ethanol series beginning at twenty

percent ethanol and ending at one hundred percent ethanol. The samples were then embedded in Spurr's resin. Thick sections of the embedded samples, of approximately two hundred and fifty nanometer thickness, were cut with a glass knife using an MT-6000 microtome. These sections were then stained with alkaline toluidine blue and examined with a light microscope for histopathological evaluation. For ultrastructural examination, ultra-thin sections of approximately ninety to one hundred nanometers were cut in the manner as the thick sections. The ultra-thin sections were placed on nickel bar grids and then stained with two percent methanol uranyl acetate followed by Reynold's lead citrate. The resulting samples were then observed using a JEOL JEM-1010 transmission electron microscope (TEM).

Histopathological Evaluation

Evaluation of heart tissue was performed using a scoring system described by Solcia et al., (1981). Cardiomyopathy was expressed as a product of the severity and the extent of the damage. Grade one severity included sarcoplasmic microvacuolations and/or inclusions, cellular edema or interstitial edema. Grade two severity included grade one severity plus sarcoplasmic macrovacuolations or atrophy, necrosis, fibrosis, endocardial lesions and thrombi. Grade 0.5 extent was defined as the presence of a single altered myocyte. Grade one extent indicated less than ten altered myocytes.. Grade two extent indicated scattered small groups of altered myocytes. Grade three extent indicated several small groups of altered myocytes. Grade four extent indicated groups of altered and confluent myocytes. Grade five extent indicated that most myocytes were affected. The data from these observations were analyzed as indicated in *Statistical Analysis*. For studying ultrastructure, representative electron micrographs from each of the rat groups

were prepared. A qualitative description of general appearance, sarcomere integrity, mitochondrial characteristics, spacing, and cellular debris was then given.

Statistical Analysis

All data are expressed as the mean ± standard error of the mean (SEM). Group means were compared by one way analysis of variance (ANOVA). Differences between groups were determined by the Bonferroni's corrected *t* test. The mean total score (MTS) for histopathological evaluations were expressed as the sum of (Severity x Extent) for each animal within a group, and then divided by the number of animals in that group. Differences in mortality proportions were assessed using the Pearson's chi-square test. A value of (P) below 0.05 was accepted as statistically significant for all analysis.

CHAPTER FOUR

Results

Survival Study

Deaths were observed in all groups during the fourteen week study (Figure 4.1). One animal died in the control group and two animals died from the ALA group. Four out of twelve animals (33%) survived in the DOX group at the end of the study. Nine out of twelve animals (75%) survived in the DOX+ALA group.

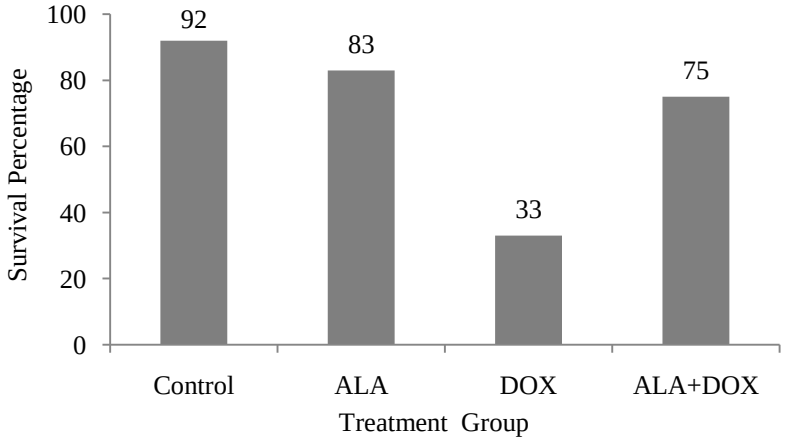

Figure 4.1 Graph illustrating the percentage of live animals at the end of the fourteen week study, per treatment group.

The proportions of rats alive at the end of the study from each group were studied as mortality percentages (Table 4.1). Person's chi-square analysis of surviving animal proportions from DOX and DOX+ALA groups showed that both are significantly different to each other, and that both groups differed significantly from the CON group (Table 4.1). P values were less than 0.001 for Pearson's chi-square tests.

Table 4.1 Parameters of cardiomyopathy evaluation at the end of the fourteen week study. CON indicates the control group. ALA indicates the group treated with α-lipoic acid. DOX indicates the group treated with doxorubicin. DOX+ALA indicates the group treated with doxorubicin and α-lipoic acid. Heart weight, heart weight to body weight ratios, and histological scores are expressed as Mean ± SEM. (*) Indicates data that are different from that of control value (P<0.001). (°) Indicates data from groups that are different from each other (P<0.001).

Parameter	CON	ALA	DOX	DOX+ALA
Mortality Percentage	8	17	67*°	25*°
Morbidity Percentage	0	0	92*°	25*°
Heart Weight (g)	0.913±0.021	0.924±0.026	0.655+0.017*	0.902±0.025
Heart Weight/Body Weight	3.885±0.054	4.019+0.052	2.782±0.065*	3.856+0.11
Histological Score	0.4±0.18	0.4±0.18	5.5±0.8*°	2.4±0.38*°

Morbidity

Effects of doxorubicin at the cumulative study dose of 15 mg per kg included ascites as a sign of morbidity, presumably due to contractile failure of the heart (Teraoka et al., 2000). Ascites was observed in eighty nine percent of the DOX group animals. In contrast, twenty five percent of the DOX+ALA animals developed ascites. No animals from the control or ALA groups showed signs of morbidity. When analyzing the morbidity proportions of all animal groups against each other, percentages were used. Animals were considered morbid at the first sign of abdominal swelling with fluid waves upon palpation. Person's chi-square analysis yielded a statistically significant difference between DOX and DOX+ALA, although both groups were significantly different from control (Table 4.1). P values were less than 0.001 for Pearson's chi-square studies.

Heart Weight

The DOX group showed significantly less mean heart weight versus the control group. The DOX+ALA group showed no significant difference from the control group. (Table 4.1; Figure 4.2). P values were less than 0.001 for ANOVA studies.

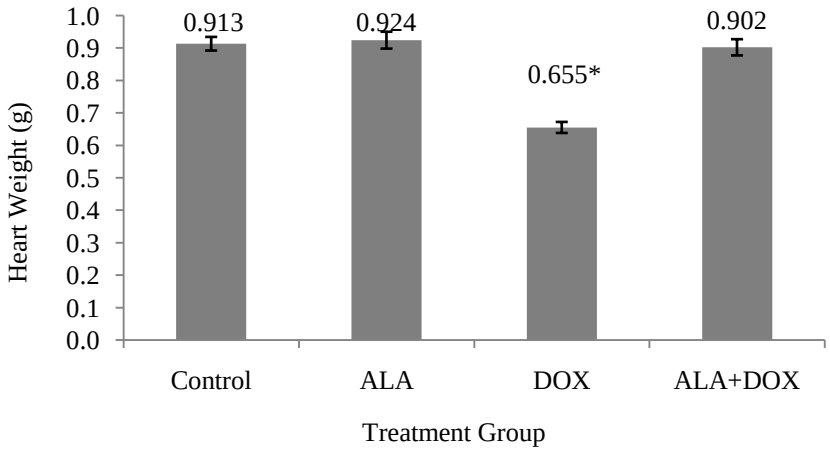

Figure 4.2 Graph of heart weight mean per treatment group. Error bars show the standard error of the mean. (*) Indicates a group mean different from that of control ($P < 0.001$).

Ratio of Heart Weight to Body Weight

Animals from the DOX group showed significantly less mean ratio of heart weight to body against the control animals. Animals in the DOX+ALA group showed no significant difference from the control group (Table 4.1; Figure 4.3). P values were less than 0.001 for ANOVA studies.

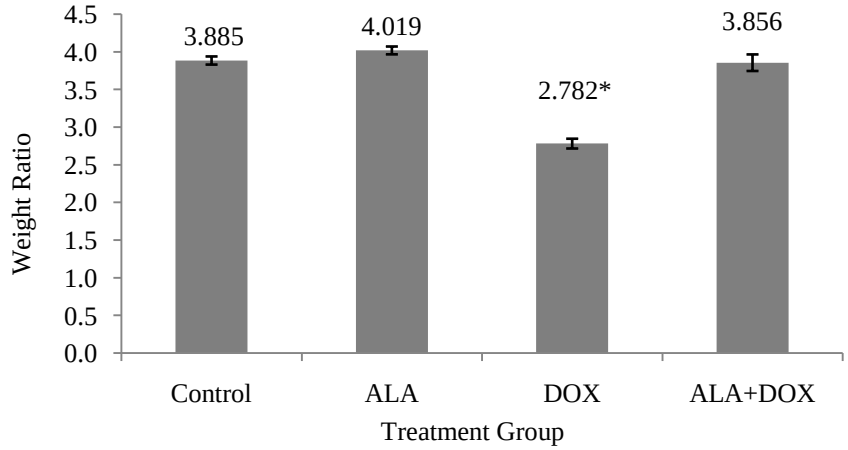

Figure 4.3 Graph of mean ratio of heart weight to body weight per treatment group. Error bars show the standard error of the mean. (*) Indicates group with a mean different from that of control ($P < 0.001$).

Histological Study

The mean histological score for the heart tissue samples gathered from DOX treated animals was 5.5 ± 0.8. The DOX+ALA group scored a mean of 2.4 ± 0.38. Both groups are statistically different from the control group, as well from each other (Table 4.1; Figure 4.4).

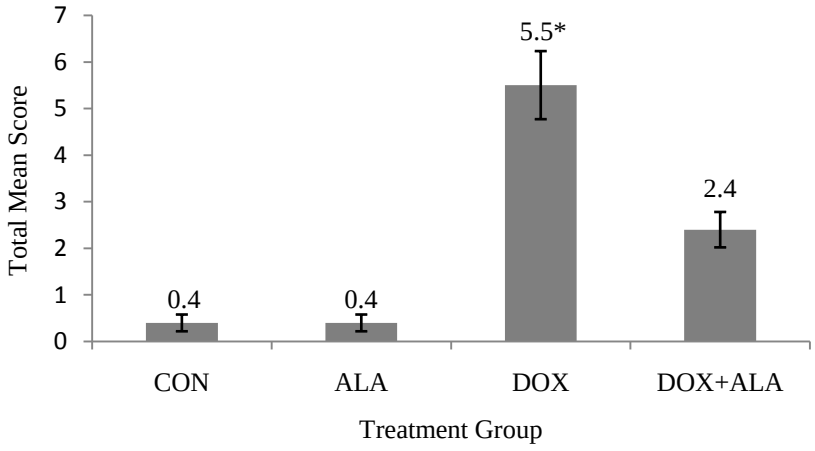

Figure 4.4 Mean histological score per treatment group. Error bars represent standard error of the mean. (*) Indicates a group mean different from that of control ($P < 0.001$).

Histological scores were created using a severity and extent evaluation of each heart tissue sample collected at the end of the study according to the methods section (Solcia et al., 1981). Prominent coalescent vacuoles were evident in all hearts in the DOX group (Figure 4.5 C). Smaller scattered vacuoles were found in the DOX+ALA group (Figure 4.5 D). Minimal myocardial vacuolization was observed in the CON and ALA groups (Figure 4.5 A; Figure 4.5 B).

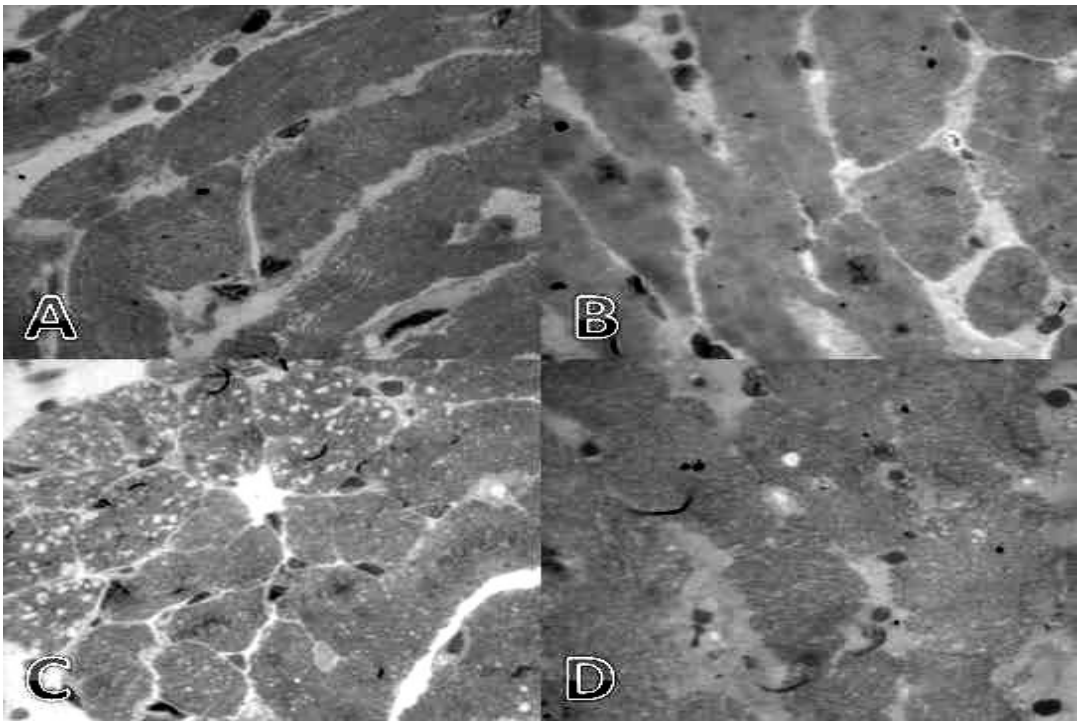

Figure 4.5 Light microscopy of rat left ventricles. (A) Control, (B) α-lipoic acid, (C) doxorubicin, (D) doxorubicin + α-lipoic acid. Hearts samples from all surviving rats at the end of the study period were examined. Representative results are shown for each group (Alkaline toludine blue, X 200).

Ultrastructural Study

Representative samples from each experimental group were studied using transmission electron microscopy (TEM). Sarcomeres appearing in the resulting

micrographs were then compared to an image-coupled schematic of a normal sarcomere (Figure 4.6).

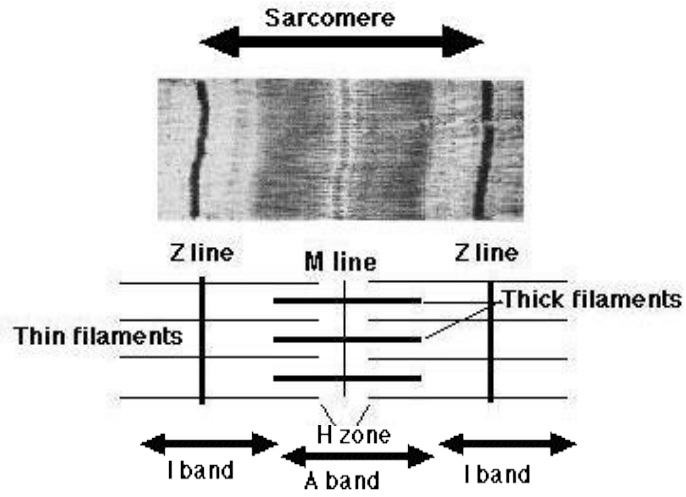

Figure 4.6 TEM image of a normal sarcomere with a basic schematic.

A control group micrograph shows characteristic myocardial tissue (Figure 4.7), including normal aspects of cellular organization and organelle structure.

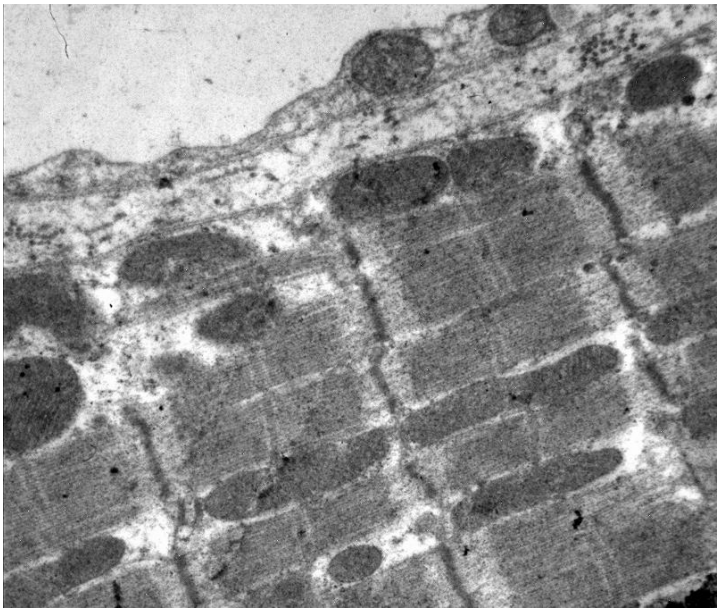

Figure 4.7 Representative micrograph of rat left ventricle control sample (Lead citrate and uranyl acetate, X 20,000).

The sarcomere schematic (Figure 4.6) serves to identify components of control cardiomyocyte sarcomeres (Figure 4.7). Control cardiomyocytes display a regular pattern of sarcomere units with clear Z disc borders. Light chain actin fibrils are seen in the I band areas of sarcomeres. Heavy myosin chains overlap the light chains in the A band areas, with areas exclusively occupied by myosin heavy chains in the H zones. Parallel M lines, representing myosin attachment structures, are clearly visible in the middle of sarcomeres units. Myofibrils show a well-defined parallel arrangement with no noticeable intervening spaces. The sarcoplasmic reticulum enveloping the sarcomere units shows no distensions, edema, or cellular debris. Mitochondria seen in the control tissue show well defined characteristics, including a regular distribution pattern of individual mitochondria lining up in rows in parallel with sarcomere chains. A regular alternation between sarcomere chains and mitochondria rows is evident. The density of mitochondria in relation to sarcomere units is homogenous. The normal mitochondrial shape is oval elongated, with some round shapes due to the cut angle. Within the individual mitochondria, cristae appear as double membrane shelves running in a rough parallel pattern. No irregularly shaped spaces were observed within the mitochondria.

The micrographs from α-lipoic acid treated rats reveal a few noticeable changes from the control group. The general organization of the cardiac tissue remains the same as control, with a well defined arrangement of sarcomere units. Z discs are well defined and the sarcomeres run in parallel chains. The I bands, A bands, H zones, and M lines are less prominently evident than in the control group. The myofibrillar structures show some disorganization, with light and heavy chains less well-defined than in the control group (Figure 4.8).

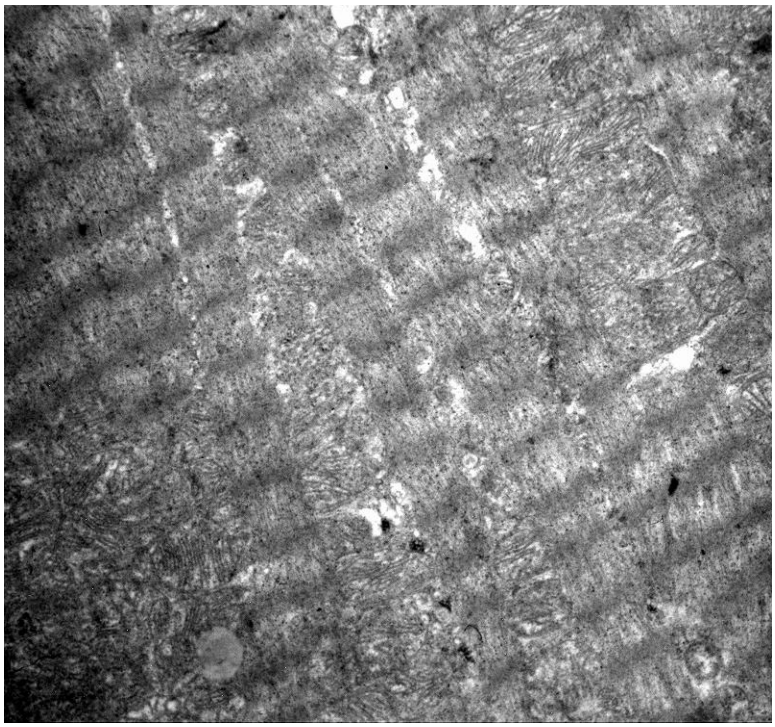

Figure 4.8 Representative micrograph of rat left ventricle treated with α-lipoic acid (Lead citrate and uranyl acetate, X 15,000).

The parallel arrangement of the myofibrils is slightly altered in a few of the sarcomeres seen, revealing irregularly shaped spaces in between. Some adjacent Z discs show a loss of parallel orientation. The sarcomere changes are not uniform in extent as these changes are in patches. As with the control micrographs, no distention of the sarcoplasmic reticulum, edema, or cellular debris is evident. Mitochondria from the α-lipoic acid treated rats retain most of their baseline characteristics. The mitochondria line up in rows parallel to the sarcomere chains as in the control group. The juxtaposition of sarcomere chains and mitochondria rows is preserved. Mitochondria density is homogenous. No alteration in the relative abundance of the mitochondria is noted (Figure 4.8; Figure 4.9).

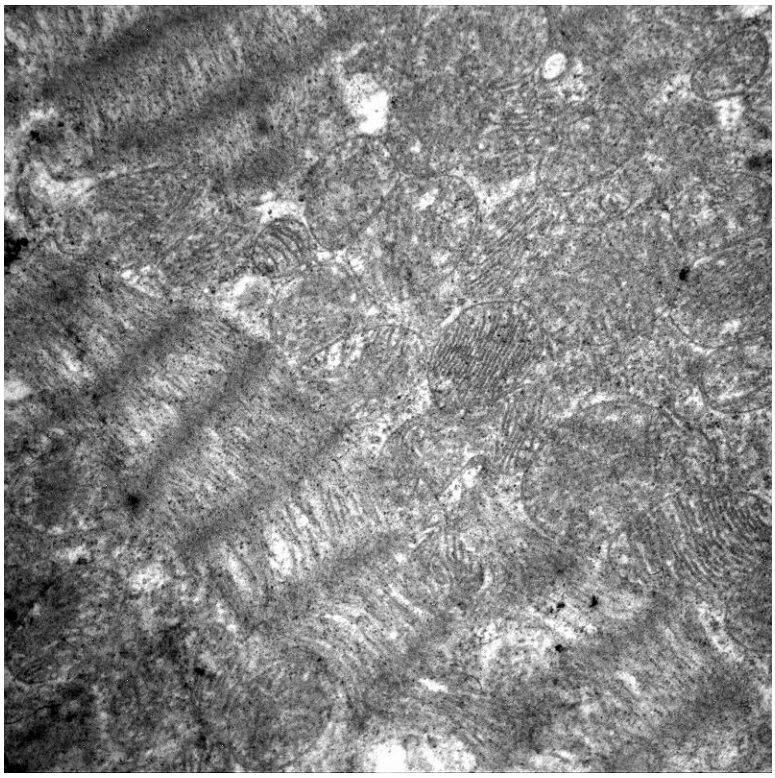

Figure 4.9 Representative micrograph of rat left ventricle treated with α-lipoic acid (Lead citrate and uranyl acetate, X 20,000).

Most mitochondria retain an oblong shape, with an increased proportion of the mitochondria exhibiting a round appearance. A noticeable change is an increase in relative size among the few round shaped mitochondria. Cristae from oblong mitochondria appear similar to that of the control group, with parallel double membrane shelves and no abnormal spaces. A few of the round appearing mitochondrial show less dense cristae and occasional irregular spacing (Figure 4.9). The structural changes observed in myofibrils and mitochondria are mild in severity and limited in extent.

Cardiac micrographs obtained from the rats treated with doxorubicin show prominent alterations from the control group. Cardiomyocyte organization is lost in all areas examined (Figure 4.10).

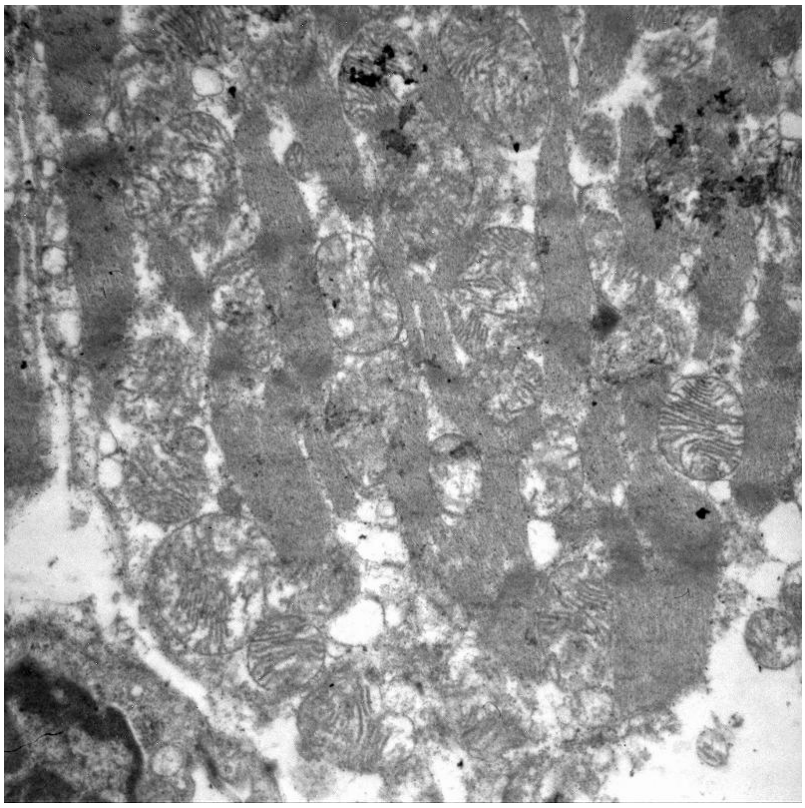

Figure 4.10 Representative micrograph of rat left ventricle treated with doxorubicin (Lead citrate and uranyl acetate, X 15,000).

A striking feature is sarcomere disorganization with loss of chain arrangement and continuity. Small islands of sarcomeres are scattered and isolated. Remaining Z discs within these sarcomere islands retain a parallel configuration. The myofibrillar arrangement is lost; heavy and light chain areas are indistinguishable from each other. The I bands, A bands, H zones, and M lines are difficult to identify. Irregular spaces are present between the disorganized myofibrils. Figure 4.11 shows a separation of the sarcoplasmic reticulum from a sarcomere island, with the resulting edematous space filled with Z disc remnants and other cellular debris.

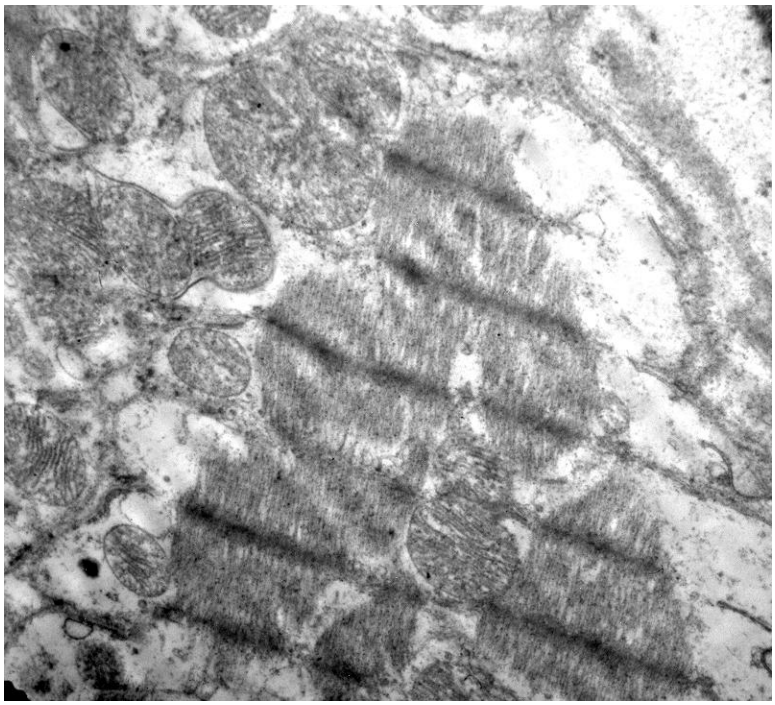

Figure 4.11 Representative micrograph of rat left ventricle treated with doxorubicin (Lead citrate and uranyl acetate, X 15,000).

Some of the spaces between sarcomere units are occupied, in a disorganized fashion, by prominently changed mitochondria. Between the loosely located sarcomeres and mitochondria, large edematous spaces containing cellular debris are evident. The density of mitochondria is variable due to clumping, but the overall abundance of mitochondria appears to be unchanged. The mitochondria show a mostly round shape in all areas examined. The relative size of the mitochondria is noticeably larger than seen in the control or α-lipoic acid groups. Within the mitochondria, cristae are greatly diminished in abundance with loss of organization and prominent irregular spacing. Many mitochondria appear as a membrane bound collection of debris, with rare cristae (Figure 4.10). Overall, TEM shows the striking severity and widespread extent of doxorubicin induced myocardial changes.

Micrographs of cardiac tissue from rats treated with doxorubicin and α-lipoic acid reveal less changes in extent and severity in comparison with doxorubicin treatment alone (Figure 4.12).

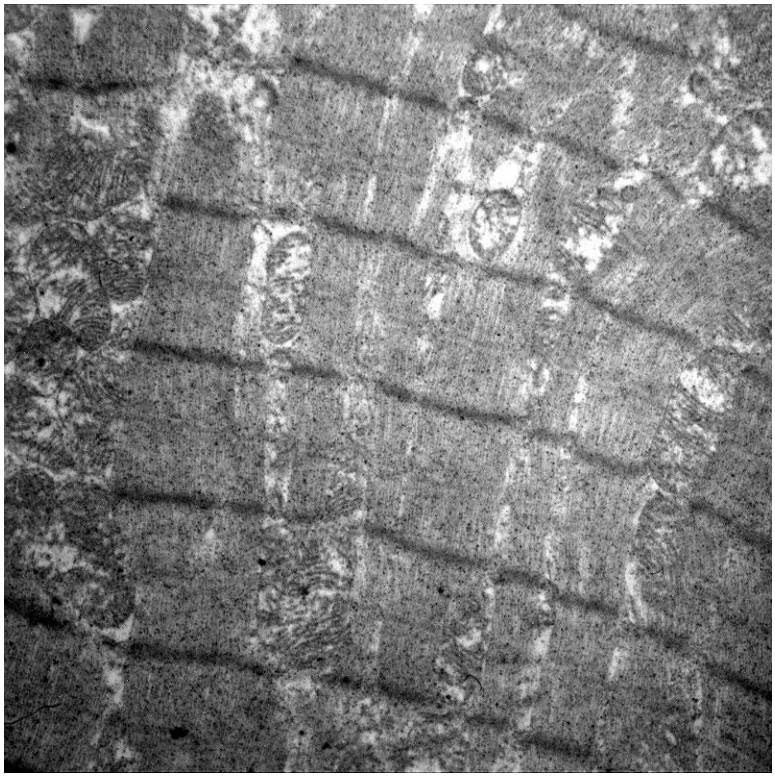

Figure 4.12 Representative micrograph of rat left ventricle treated with doxorubicin and α-lipoic acid (Lead citrate and uranyl acetate, X 15,000).

Cardiomyocytes retain a recognizable degree of organization. Sarcomere units maintain a chain like arrangement with notable continuity. Clear Z discs are in parallel and define areas of evident myofibrillar orientation. Myofibril heavy and light chains run in parallel with some areas of irregularity and spacing. In most of the sarcomeres examined, the I bands, A bands, H zones, and M lines are recognizable. Sarcomere chains maintain a parallel arrangement with a few exceptions. Some spacing between sarcomere strands is present and contains changed mitochondria along with some cellular

debris. Separation of the sarcoplasmic reticulum from sarcomeres can be seen (Figure 4.13), although to a lesser extent than observed in the doxorubicin group.

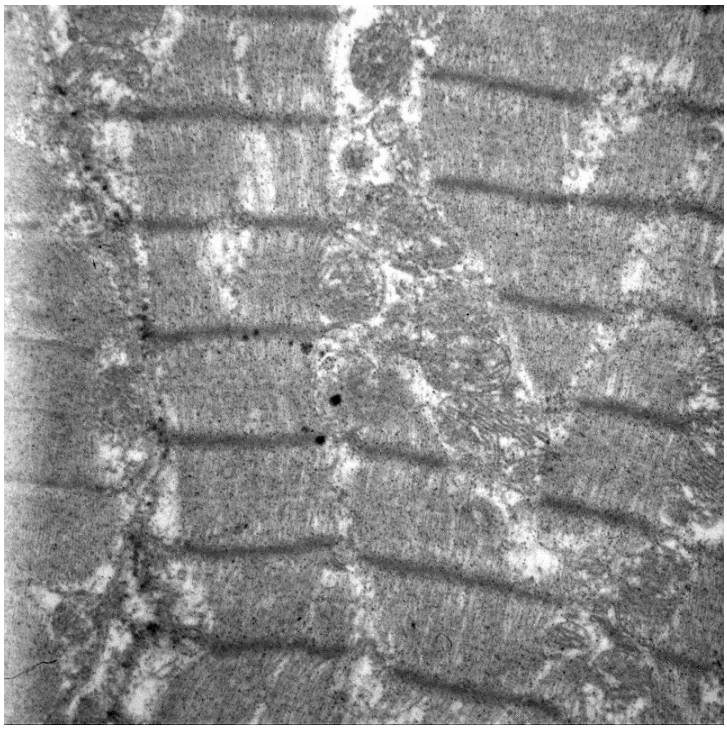

Figure 4.13 Representative micrograph of rat left ventricle treated with doxorubicin and α-lipoic acid (Lead citrate and uranyl acetate, X 15,000).

The mitochondria are arranged is in rows between sarcomere chains, but are less uniformly distributed than in control tissue; some clumping is evident with the intervening areas occupied by cellular debris. Mitochondria vary in shape from oblong to round, and few show evidence of swelling. Clusters of mitochondria with various degrees of change are noted. The most prominently changed mitochondria show a near complete loss of cristae with debris-filled spaces. The most preserved of the mitochondria show little loss of cristae, with parallel double membrane shelves and few spaces present. Most mitochondria are evenly spaced in a continuum between both degrees of damage severity.

CHAPTER FIVE

Discussion

Doxorubicin is a well known inducer of cardiomyopathy in rats. The mechanism of cardiomyopathy begins with a one-electron redox cycling of doxorubicin in the mitochondria (Minotti et al., 1990). The free radicals generated by this reaction are propagated through a membrane lipid peroxidation cascade (Morrow et al., 1992). The results are cytotoxic aldehydes which diffuse within the cell and cause impaired energy production, protein and DNA damage, inflammation, and necrotic or apoptotic death of cardiac cells (Luo et al., 1997). The major endogenous defense against free radicals in the cardiomyocyte is glutathione (GSH). Doxorubicin depletes GSH in the cardiomyocytes, which leaves the heart vulnerable to free radical attack (Paranka and Dorr, 1994). Attempts to protect the heart from doxorubicin induced cardiomyopathy focused on antioxidants. Antioxidants attenuate free radical cascades by donating reducing electrons (Mannervik, 1985). Endogenous antioxidant mimics demonstrated that doxorubicin induced damage can be attenuated *in vitro* (Eugene et al., 1999). Exogenous antioxidants showed different degrees of success, depending on the availability of the antioxidant in lipid and aqueous phases of the cell. Melatonin, an amphipathic antioxidant, demonstrated effective attenuation of doxorubicin induced cardiomyopathy (Morishima et al., 1998). Of the freely available antioxidants, α-lipoic acid shares its amphipathic properties with melatonin, but has several direct and indirect mechanisms of antioxidant protection (Bast and Haenen, 1990; Packer and Tritschler, 1996; Chevion et al., 1997; Han et al., 1997; Nohl and Gille, 1998). Very few studies

have biochemically measured the extent of cardiotoxicity attenuation with α-lipoic acid, and such studies showed promising results (Balachandar et al., 2003; Al-Majed et al., 2002). The rational of this study thus emerges, attempting to explore aspects of α-lipoic acid protection previously unexamined in doxorubicin treated rats, namely evidence of histological and ultrastructural protection.

Using a rat model of doxorubicin induced cardiomyopathy, this study analyzed different aspects of doxorubicin cardiotoxicity, along with any protection offered by α-lipoic acid. Mortality from doxorubicin is the end result of heart failure in rats (Matsui et al., 1999). The chief mechanism of cardiomyopathy seems to arise from contractile failure after a prolonged oxidative injury to cellular organelles and macromolecules (Balachandar et al., 2003). Compromise of cardiac function is evident by the presence of ascitic fluid in the peritoneum, a significant indicator of contractile heart failure (Teraoka et al., 2000). At the organ level, cardiac mass is affected by impairment of contractile protein synthesis and by DNA damage. This leads to cardiomyocyte necrosis or apoptosis, therefore contributing to loss of heart mass (Botton et al., 1998). Heart weight and heart weight to body weight ratios were assessed in order to evaluate whole organ toxicity from doxorubicin. At the cellular level, intracytoplasmic damage is marked by dilated sarcoplasmic reticulum. This dilation leads to the formation of vacuoles within cardiomyocytes, which displace lost contractile structures. Vacuole formation is the hallmark of doxorubicin cardiotoxicity (Aversano and Boor, 1983). Quantification of vacuole size and abundance serve to assess the degree of cellular toxicity in study rats.

Compared to the control animals in this study, animals treated with a total of 15 mg per kg doxorubicin showed significantly increased mortality and morbidity, along

with significantly decreased heart weight and heart weight to body weight ratios. Histological changes in response to doxorubicin administration were prominent in study rats, as evident in significant vacuole formation in cardiomyocytes. This is an expected result and is consistent with changes observed by Morishima et al. (1998). As mentioned previously, the generation of reactive oxidative radicals is the underlying mechanism of doxorubicin cardiotoxicity in rats (Minotti et al., 1990).

As an antioxidant, α-lipoic acid works as a direct inactivator of free radicals by electron donation (Packer and Tritschler, 1996), bolstering endogenous enzymatic defenses against oxidative stress (Bast and Haenen, 1990; Han et al., 1997), and by regenerating other antioxidants within the cell (Kegan et al., 1992; Nohl and Gille, 1998). Administration of 64 mg per kg total dose of α-lipoic acid in rats treated with doxorubicin resulted in a statistically significant reduction in mortality in this study, although mortality in these animals was still higher than in control rats. One death was observed in the control group, and two in the α-lipoic acid group. One possible cause of such deaths may be from inadvertent trauma caused by intra-peritoneal injections. The significant reduction in mortality offered by α-lipoic acid in doxorubicin treated rats is due to attenuation of cardiac failure, and is in agreement with a similar study using melatonin as the antioxidant (Morishima et al., 1998).

Morbidity proportions related to doxorubicin, as assessed by ascites formation, were reduced in a statistically significant degree by α-lipoic acid co-administration in this study. Although ascites in these rats was not reduced to control levels, this reveals a marked improvement in cardiac function status. Preservation of cardiac contractile force mitigates the formation of abdominal ascites, the hallmark of cardiac failure. This is also

in agreement with Morishima et al. (1998). The preservation of cardiac function offered by α-lipoic acid is a measure of its antioxidant effect, and is supported by biochemical evidence in cardiac tissue co-treated with doxorubicin (Balachandar et al., 2003; Al-Majed et al., 2002).

At the gross level, this study examined the effect of doxorubicin on heart weight, a reliable cardiomyopathy indicator (Morishima et al., 1998). Heart weights from animals treated with doxorubicin were significantly reduced from control levels, while co-administration of α-lipoic acid maintained heart weight to a level similar to that of control rats. Standardizing heart weight against total body weight minimizes the compounding factor of animal growth rate change due to treatment. The ratio of heart weight to body weight was preserved in doxorubicin treated animals when α-lipoic acid was administered, with ratios similar to that of control animals. This indicates a preservation of cardiac muscle mass in rats treated with combined doxorubicin and α-lipoic acid. Morishima et al. (1998) demonstrated a similar effect when using melatonin as a cardioprotectant. Cardiac mass preservation points to decreased doxorubicin induced oxidative destruction of cellular components, including myofibrillar proteins (Luo et al., 1997).

Histological examination of doxorubicin treated cardiomyocytes shows a characteristic pattern of structural damage. Doxorubicin treatment results in myofibrillar loss and subsarcolemmal bleb formation (Eugene et al., 1999). Spaces resulting from structural loss coalesce into vacuoles visible in light microscopy, and the emerging vacuoles represent the end result of cytological damage caused by free radical cascades (Aversano and Boor, 1983). Vacuoles are a reliable method of evaluating cellular

damage since it is quantifiable. This study evaluated histological damage by scoring vacuole size and abundance as devised by Solcia et al. (1981). Doxorubicin treatment induced abundant macrovacoule formation in all hearts exposed to the agent. Co-treatment with alpha α-lipoic acid, at 64 mg per kg total dose, resulted in smaller and less numerous vacuoles. The striking reduction in doxorubicin induced vacuoles seen with α-lipoic acid is similar to the results of a similar study using melatonin to attenuate doxorubicin induced histological changes (Morishima et al., 1998). The biochemical marker evidence of doxorubicin free radical protection by α-lipoic acid (Balachandar et al., 2003; Al-Majid et al., 2002) supports the histological findings of this study, especially since vacuole formation is a reliable sign of severe oxidative damage in the cell (Aversano and Boor, 1983).

Ultrastructural studies using transmission electron microscopy (TEM) on doxorubicin treated tissue show characteristic changes. Previously, TEM analysis on heart biopsy samples from humans treated with doxorubicin showed severe myofibril loss with sarcotubular dilations coalescing into vacuoles (Troti et al., 1986). Such changes mirrored the light microscope changes seen by Eugene et al. (1999). In this study, doxorubicin induced similar changes in the rat heart. Loss of sarcomere arrangement with prominent disorganization and loss of myofibrils was evident in this study. Van Vleet et al. (1980) demonstrated with TEM that in the dog heart, doxorubicin induces the formation of giant mitochondria alongside scattered filaments and distended sarcoplasmic reticulum. Similar changes are seen in this study as doxorubicin induced marked mitochondrial enlargement with loss of cristae organization. Spaces delineated by sarcoplasmic reticulum, and filled with cellular debris, were also seen in cardiomyocytes

exposed to doxorubicin in this study. The widespread loss of contractile elements along with the mitochondrial degeneration induced by doxorubicin supports previous work (Luo et al., 1997; Praet and Ruysschaert, 1993) and points to the ultrastructural underpinning of cardiac contractile failure with doxorubicin. The loss of myofibrils and sarcomere breakdown also support the findings of gross cardiac mass loss observed in this study and as seen by Morishima et al. (1998). Micrographs of cardiomyocytes exposed to α-lipoic acid show mild myofibril loss and disorganization in scattered area. This is a paradoxical finding since α-lipoic acid has been found to have no side effects (Hagen et al., 1999). This finding may be an idiosyncratic effect of α-lipoic acid, possibly due to an interaction with the delivery vehicle. Further studies to examine the safety of α-lipoic acid in delivery vehicles is thus clearly warranted.

When α-lipoic acid is co-administered with doxorubicin, the resulting micrographs show a noticeable degree of ultrastructural preservation. Sarcomere units largely retain their orientation and continuity. Myofibrillar loss is less pronounced with a noticeable preservation of banding patterns. Swollen and deformed mitochondria (Megamitochodria) are less noticeable than in the doxorubicin group, and cristae exhibit overall improvement in density and organization. No previous studies have examined the ultrastructural effects of combined doxorubicin and α-lipoic acid in cardiomyocytes. Interaction of doxorubicin and α-lipoic acid has only been studied ultrastructurally by Prahalathan et al. (2006) in rat testicular tissue, in which they demonstrated no change from control with concomitant doxorubicin and α-lipoic acid treatment. It should be noted that rat testicular cells have no sarcomeres and may accumulate and metabolize doxorubicin and α-lipoic acid in a different manner.

The findings of Prahalathan et al. (2006) offer partial support to the current findings. Although this study demonstrates that α-lipoic acid offers a pronounced degree of ultrastructural cardiac protection against doxorubicin induced changes, further work in this area is necessary.

In conclusion, the findings of this study support the hypothesis that α-lipoic acid offers protection from doxorubicin induced cardiomyopathy in rats. All studied indicators of cardioprotection showed improvement. Cardiac mass preservation was the strongest finding in this study, with α-lipoic acid showing remarkable protection of doxorubicin induced cardiac tissue loss. In terms of survival, morbidity, and mortality, the protective action of α-lipoic acid was statistically significant, although this improvement did not reach complete reversal with the dosages administered. Histological analysis shows that α-lipoic acid exhibited a striking degree of protection in doxorubicin treated rat hearts. Transmission electron micrography provided a remarkable look at the ultrastructural details which support and offer further insight to the findings of this study.

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