METABOLISM OF RETINOL IN ADRIAMYCIN-INDUCED CARDIOMYOPATHY

A Thesis Presented to the Faculty of Graduate Studies in
Partial Fulfilment of the Requirements for the Degree of:

MASTER OF SCIENCE IN PHYSIOLOGY

By

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of Master of Science

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I. ABSTRACT

Adriamycin (Doxorubicin), a potent antitumor drug, has been widely used in the treatment of a variety of solid and soft tumors. However, its therapeutic use is limited by the development of a dose-dependent cardiomyopathy which can lead to the therapy-resistant congestive heart failure. Free radical generating properties of adriamycin are considered to play a major role in the pathogenesis of this side effect, thus highlighting the potential role of endogenous antioxidants including vitamin A in protection against adriamycin-induced cardiomyopathy. In this study, we have examined the metabolism of vitamin A (retinol) in adriamycin-induced cardiomyopathy in the rat model.

Adriamycin was administered in 6 equal i.p. injections over a period of two weeks (cumulative dose 15 mg/kg). Three weeks after the treatment, retinol and ^{3}H-radio labeled retinol were measured in heart, liver, kidney and plasma using HPLC method. In the storage organs, the liver and kidneys, stored amounts of vitamin A were assessed by measuring the levels of retinol palmitate. Activities of retinol mobilizing enzymes, bile salt-dependent retinol ester hydrolase (BSD-REH) and bile salt-independent retinol ester hydrolase (BSI-REH) were also examined in the liver. Levels of α-tocopherol, another known endogenous nonenzymatic antioxidant, were also examined using HPLC method. All of the results were compared with the matching controls.

Usage of this treatment protocol caused cardiomyopathy and congestive heart failure. Treated animals showed labored breathing, ascites (63.6 ± 10.1 ml) and high mortality. Intraventricular peak systolic pressure was 91.1 ± 10.2 mmHg which was 25% less than the

control values. A significant rise (410%) in the end diastolic pressure 29.6 ± 3.2 mmHg was noted in the treated group. Retinol levels in the heart and the plasma in the adriamycin-treated group (ADR) were unchanged or slightly decreased compared to the control (CONT). Retinol and retinol palmitate (storage form of retinol) were significantly decreased in the liver of cardiomyopathic animals. Kidney retinol and retinol palmitate concentrations in the treated group were found unchanged or slightly decreased. Radio-labeled retinol levels were increased in the heart, plasma, liver and kidneys of the adriamycin-treated animals, whereas BSD-REH and BSI-REH activities were found decreased in the adriamycin group. Alpha-tocopherol levels were found to be increased in plasma and liver of adriamycin-treated animals, while a decrease was noted in the kidneys. The concentration of α-tocopherol in the heart was found to be unchanged or slightly higher.

This study shows that retinol levels were strictly maintained in the heart and plasma, even in the severe failure stage. This may be due to the increased mobilization from the storage sites in the liver. Increased consumption in the heart was indicated by an increase in the radio-labeled retinol in this organ as well as in the plasma. The study shows that under condition of increased myocardial oxidative stress, there is an increased consumption of antioxidant vitamin A.

II. INTRODUCTION

Adriamycin (Doxorubicin), an antineoplastic anthracycline, is widely used in the treatment of a large number of soft and solid tumors. Its usage is limited by the development of a number of acute and chronic effects. Acute side effects develop soon after the beginning of the treatment and are characterized by the clinically manageable myelosuppression, nausea, vomiting and arrhythmias. The more serious side effect, connected with the administration of this drug is the development of cardiomyopathy and ultimately congestive heart failure. Patients with these chronic effects are found to be refractory to inotropic and mechanical assist devices. The morphological changes in cardiac cells, that are induced by adriamycin administration, include cytoplasmic vacuolization, distention of sarcoplasmic reticulum, loss of myofibrils, disruption of sarcomeres and swelling and lysis of mitochondria.

A number of different mechanisms are thought to be involved in the development of this drug-induced pathological state. Many studies have shown the involvement of oxidative stress in adriamycin-induced cardiomyopathy. This is also supported by the fact that the usage of different antioxidants such as probucol, vitamin E, trolox, melatonin and vitamin A has provided a certain degree of the protection against the development of cardiomyopathic damage.

Although vitamin A has been shown to offer some protection against this pathological heart condition , its metabolism during adriamycin-induced cardiomyopathy has never been examined. The present study was undertaken in order to examine the metabolism of vitamin A during the development of adriamycin-induced cardiomyopathy.

Adriamycin cardiomyopathy was induced in rats using repetitive injections of the drug in a regime previously established in our laboratory. Clinical signs and hemodynamic assessments were performed in order to establish the presence of heart failure. Retinol levels were measured in heart, plasma, liver and kidneys of adriamycin-treated animals. The activity of retinol mobilizing enzymes, bile salt-dependent and bile salt-independent retinol ester hydrolases was measured in the liver. The storage forms of retinol (retinol palmitate) were also measured in the liver and kidneys. The levels of another endogenous antioxidant vitamin, α-tocopherol (vitamin E), were also assessed in plasma, heart, liver and kidneys of the adriamycin-treated animals. Radio-labeled retinol was used to follow the mobilization of retinol in these animals.

Even though there was no significant change in total levels of retinol in the heart and plasma, study using radio-labeled vitamin A revealed the mobilization of retinol from its storage organs such as the liver. Increased uptake of radio-labeled vitamin A in the heart of adriamycin-treated animals, strongly indicates the increased utilization of vitamin A in these organs under oxidative stress. The present study shows that the metabolism of vitamin A even, in severe pathological conditions such as adriamycin-induced cardiomyopathy and heart failure, is closely regulated.

III. LITERATURE REVIEW

A. Vitamin A

A.1. Brief history

One of the first clearly documented symptoms of nutritional deficiency in human history was the development of night blindness. The first scientifically recognized description of vitamin A dates as early as 1909 when Steep reported the finding of a life-essential compound in egg yolk (Steep, 1909). Soon after this discovery, McCollum and Davis reported a lipid-soluble dietary factor and rat-growth promoter, which they named "fat soluble fraction A" (McCollum and Davis, 1913). Extensive population studies have proved the link between vitamin A deficiency and night blindness. This fat soluble fraction A was finally named vitamin A in 1920 by Drummond (1920).

In 1930, Karrer and associates determined the chemical structure of the provitamin A compound, β-carotene and one year later, the chemical structure of vitamin A (retinol) was published (Karrer, 1931). The studies which determined the physiological role of vitamin A in the visual processes earned Wald a Nobel Prize (Wald, 1936 a,b). In 1937, the first isolation of vitamin A from fish liver was accomplished (Holmes and Corbert, 1937) which was followed by the chemical synthesis of retinol a few years later (Arens and Van Dorp, 1946).

A.2. Chemical structure and properties of vitamin A and its related compounds

Vitamin A is a broad, generic term that describes any compound possessing the biological activity of retinol. The broader term "retinoids" includes both naturally occurring

forms of vitamin A and synthetic analogs of retinol, with or without biological activity (IUPAC-IUB Joint Commission on Biochemical Nomenclature: Nomenclature of retinoids, Recommendations, 1982). The parent compound for such a broad group is all trans-retinol, a polyisoprenoid molecule containing a cyclohexenyl ring, with a molecular weight of 286 (Blomhoff, 1994) (Fig. 1).Other biologically active forms of vitamin A are retinal (aldehyde form active in vision) and retinoic acid (a potent transcription factor) (Fig. 1). Retinol is stored in a biologically inactive form as retinyl-esters (Fig. 1). Retinyl palmitate is the ester form of vitamin A with a long chain fatty acid and in higher animals and humans it is generally found in storage organs such as the liver and adipose tissue (Wake, 1980) (Fig. 1).Only plants and higher photosynthetic microorganisms are capable of *de novo* synthesis of vitamin A (Goodwin 1963; 1971). This means that higher animals must obtain vitamin A from their diet in an uptake process that is highly regulated, in order to keep vitamin A levels in a narrow range span and prevent possible toxicity of free retinol (Palace et al., 1999b). The major natural sources of vitamin A in a diet include certain plant-derived carotenoid pigments, such as all trans β-carotene and the long chain retinyl esters found in animal tissues.

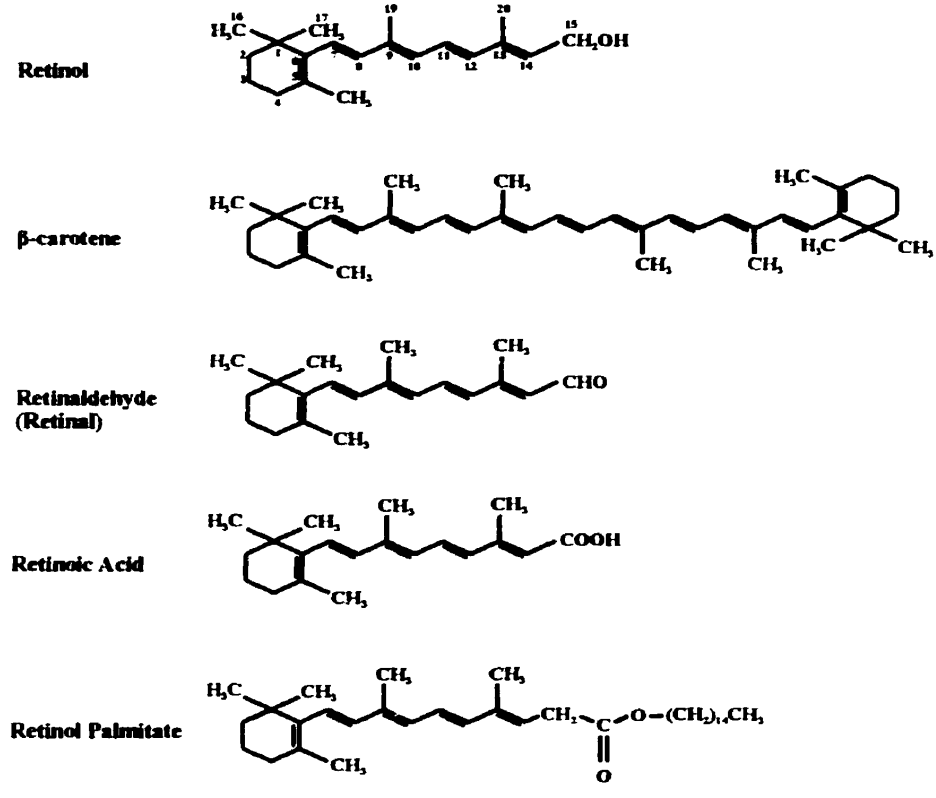


Figure 1: Chemical structure of retinol and related forms.

A.3. β-carotene and its conversion to retinol

Beta-carotene is a yellow pigment that consists of two molecules of retinal joined at the aldehyde end of their carbon chains (Fig. 1). One molecule of β-carotene, through a central cleavage pathway, produces two molecules of retinal that are then converted into retinol, whereas the eccentric cleavage would also result in the production of additional β-apo-carotenal molecule which is then converted to retinoic acid through β-apo-carotenoid acid (Dmitrovski, 1991).

Retinal concentration is low in all tissues except retina due to high reaction kinetics. Retinal is reversibly converted back to retinol or irreversibly converted to another active form, retinoic acid, which is an important transcription factor. This process is mediated by the activity of a hydrolytic enzyme, retinal oxidase. It is also suggested that retinol can undergo the direct conversion to retinoic acid without going through intermediate steps of retinal production (Blomhoff et al., 1990) (Fig.2).

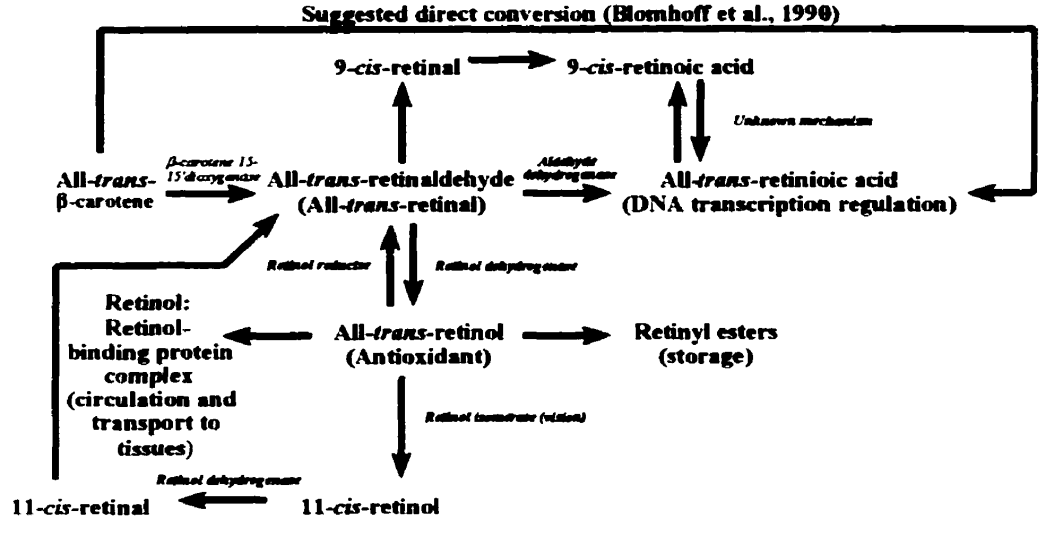


Figure 2: Pathways for the conversion of β-carotene into active retinoid metabolites.

A4. Physiological functions of Retinol

A.4.a. Role of retinol in vision. Retinol is known to play a crucial role in vision, due to its chromaphoric nature. The conversion of light stimulus into a neurological signal is achieved by the activity ot a photon-initiated enzyme, photo-isomerase and an opening of the fast calcium ion channels in the rod cells of the retina, which triggers the cell membrane depolarization causing the production of nerve impulses (Saari, 1994; Wald, 1968).

A.4.b. Retinol in the regulation of DNA transcription. Another important biologically active form of vitamin A, retinoic acid, is bound to retinoic acid binding protein (RABP) and transported into the nucleus. In the nucleus, it non-covalently binds to a number of nuclear receptors (RAR and RXR type) that regulate the expression of several genes that are involved in the control of cellular proliferation and differentiation (Clawitter et al., 1990; Blomhoff, 1994; Mangelsdorf, 1994; Olson, 1994; Chambon, 1996; Ross, 1998).

A.4.c. Other probable beneficial role of retinol. In addition to the regulation of gene expression, the antioxidant properties of retinol and retinoids (Krinsky, 1988; Palozza and Krinsky, 1992; Singal et al., 1998; Palace et al., 1998; 1999; Livrea and Tesoriere, 1998; Keys and Zimmerman, 1999), have the potential for the treatment and prevention of cardiovascular (Street et al., 1994; Morris et al., 1994; Odetti et al., 1984) and other diseases (Heller at al., 1985; Winkelmann et al., 1983, Suharno et al., 1993, Sharma et al., 1986, Comstock et al., 1997; Honkanen et al., 1989,Ward, 1994, Omenn et al., 1996). Vitamin A has also been shown to improve resistance to infectious diseases and decrease the general mortality in population (Tee, 1992; Bates, 1995; Glasziou and Mackerras, 1993; Olson, 1987).

A.5. Dietary sources and daily requirements

Retinoids are generally found in the dietary products such as carrots, tomatoes, sweet potatoes, yellow and green leafy vegetables, cheese, milk products and animal meat (Romieu et al., 1990). One international unit of vitamin A is defined as 0.3 μg of all trans-retinol. However, for nutritional purposes the more common measurement is RE (retinol equivalents), which converts both vitamin A and carotenoids into a single unit. One retinol equivalent is

equal to 6 μg of β-carotene or 12 μg of mixed dietary carotenoids (IUPAC-IUB Joint Commission on Biochemical Nomenclature. Nomenclature of Retinoids, 1982). Daily requirements of vitamin A are still a matter of debate in several countries. However, the generally recommended daily dose is 1000 RE (retinol equivalents) for adults and 375-700 RE for infants (National Academy of Sciences, US Food and Nutrition Board).

A.6. Metabolism of vitamin A

Retinol is an essential nutrient for humans and the majority of vertebrates (Blomhoff, 1994; Olson, 1994; Ross, 1998). The intake is from two main dietary sources: carotenoids and retinyl esters. Retinyl esters are hydrolyzed into retinol inside the intestinal lumen prior to their absorption (Rigtrup et al., 1994). This is achieved by the hydrolytic action of pancreatic lipases such as carboxyl ester lipase and retinyl ester hydrolases (Harrison, 1993; Erlanson and Borgstrom, 1968). Absorption as well as cleavage of retinol from its esters is fully dependent on the concentration of retinol itself (Dew and Ong, 1994, Blomhoff, 1994, Blaner and Olson, 1994).

It is known that the liver represents a major regulatory organ for the metabolism of vitamin A. Chylomicron remnants are further sequestrated by the lipolytic processes in the liver's space of Disse where they are solely absorbed by the hepatocytes. This absorption is achieved by the receptor-facilitated uptake process that is mediated by the low-density lipoprotein receptor (LDL-R) and receptor-related transport protein (LPR). Soon after the absorption into the early endosomes, retinyl esters are rapidly hydrolyzed by the action of bile salt-independent retinol ester hydrolase (BSI-REH). Small quantities of bile salt-dependent retinol ester hydrolase (BSD-REH) are also found in hepatocytes.

Hydrolyzed retinol is first bound to the cellular retinol binding protein (CRBP) type I, which is rapidly transported into the endoplasmic reticulum (ER) (Blomhoff et al., 1985). Prior to the uptake into the endoplasmic reticulum, retinol is released from its complex with CRBP type I. Blaner (1991) has reported that hepatic ER contains the largest concentration of retinol specific binding proteins (apo-RBP). These proteins bind retinol and transport it to the Golgi apparatus where it is prepared for secretion (Blaner, 1989). From this point, the RBP- retinol complex can be stored in liver stellate cells or can be secreted back into circulation. The amount of newly endocytosed retinol that will be secreted directly into circulation depends on the general vitamin A status and this can be used as a good indicator of overall nutritional status (Batres and Olson, 1987).

A.6.a. Storage in liver. Retinol intended for storage is rapidly transported into the presinusoidal stellate cells. This mechanism is reversible and tightly regulated by RBP, since the stellate cells can only uptake retinol already bound to RBP (Blomhoff et al., 1982; 1984; 1985; Gjoen et al., 1987; Blaner et al., 1985). The paracrine transport of RBP-retinol complex to the stellate cells is rapid (Blomhoff et al., 1988). Stellate cells store retinol in a form of retinyl esters in the intracellular lipid droplets (Wake, 1971), which are formed through vacuolization of the rough endoplasmic reticulum. These droplets exist in membrane bound and unbound forms (Wake, 1980). Stellate cells are also found to be rich in cellular retinol binding protein type I (CRBP I) and hydrolytic enzymes (BSD-REH, BSI-REH). Both LRAT and retinoic acid binding protein type I (CRABP I) are abundant in these cells. High concentration of CRABP I indicates the regulation of the expression by retinoic acid of both CRBP type I and LRAT (Smith et al. 1991; Matsuura and Ross, 1993). When needed, retinyl

esters stored in stellate cells, can be hydrolyzed by BSI and BSD-REH, complexed with RBP and then transported out of the cell. Previous studies suggested that retinol first had to be transported from the stellate cells into the hepatocytes (sight of RBP production) and then secreted bound with RBP. However, recent studies suggest that stellate cells in serum free cultures can independently produce RBP (Andersen et al., 1992). This could allow secretion of RBP-retinol complexes directly into circulation, where it binds to another liver-produced plasma protein thransthyretin (TTR), also known as prealbumin (Noy et al., 1992; Shankar and De Luca, 1988). The TTR-RBP-Retinol complex has a high molecular weight that reduces glomerular filtration of otherwise small and filterable retinol molecule (Palace et al. 1999b).

A.6.b. Retinol binding protein (RBP): structure and function. This important member of the lipocalin protein family has a single polypeptide chain with a molecular weight of 21,000 and binds with all trans-retinol in a 1:1 ratio (Kanai et al., 1968). The biochemical structure of RBP provides it with a special hydrophobic pocket for the transportation of retinol (Newcomer and Ong, 1990). In plasma, the RBP-retinol complex is reversibly bound with TTR. Despite previous beliefs that the liver is the only site of RBP secretion, new studies suggest that a number of extrahepatic tissues are also capable of producing and secreting this transport protein (Blomhoff, 1994; Blaner, 1989).

A.6.c. Retinol turnover and recycling in plasma. The previous belief that retinol is taken up in the tissues and irreversibly utilized is contradicted by the recent reports that retinol actually recycles between the plasma, liver and extrahepatic tissues (Green and Green, 1994). In fact, it is now thought that majority of retinol leaving the plasma is recycled

several times, since the plasma turnover rate is more than an order of magnitude greater than the utilization rate (Green et al. 1985).

A.6.d. Retinol absorption by extrahepatic tissues. The exact mechanism through which peripheral cells absorb retinol still remains to be defined. Studies based on a comparison of *in vitro* RBP-retinol dissociation rates and cellular retinol uptake rates suggest that retinol first dissociates from the complex with RBP. It is then taken into cell membranes where it binds to the CRBP (Noy and Xu, 1990; van Bennekum et al. 1993; Levin et al. 1993). Several authors have also reported the internalization of RBP, and even TTR in different cell types, which indicates a specific role for these transport proteins in the uptake of retinol into the extrahepatic tissues (Divino and Schussler, 1990).

A.6.e. Metabolism of retinol in extrahepatic tissues. Once taken into the extrahepatic tissues, retinol follows one of two distinctive pathways. In the first, retinol is esterified with fatty acids by the activity of LRAT and stored in cells in the form of retinyl esters. Stored retinol can be mobilized from its esterified form by the activity of REH. In the second pathway, however, retinol is converted into its active metabolite-retinoic acid. Immediately after its synthesis, the retinoic acid binds to its specific binding protein, retinoic acid-binding protein (RABP), which mediates transport of retinoic acid to its functional sites in the nucleus.

A.6.f. Catabolism. Studies using radioactive urinary, biliary and fecal metabolites have proposed several different catabolic mechanisms. Cytochrome P-450 is thought to be involved in the oxidation of retinol to 4-hydroxy and 4-oxo retinol (Leo and Lieber, 1985), which are the metabolites found in rat liver microsomes. Other authors have proposed that

retinol is excreted through bile and urine, via the formation of glucuronides (Barua and Olson, 1986).

B. Role of Vitamin A and Carotenoids in Cardiovascular Pathology:

A large number of epidemiological studies have been performed, primarily to establish a correlation between retinol plasma concentration, retinoid intake and development and progression of cardiovascular disease (Palace et al., 1999b).These population studies have yielded conflicting data.

B.1. Supportive studies

In the last twenty years, a large number of population studies have supported the positive correlation between the increased level of beta carotene and retinol in a healthy population and decreased incidence of cardiovascular deaths and diseases (The Israeli Dietary Monitoring Study, The Western Electric Study, The Prospective Basel Study, Palgi, 1981; Riemersma et al., 1991). The Finnish population is generally excluded from the cross-cultural, cardiovascular epidemiological studies, due to the possible genetical susceptibility for the development of heart disease, which is independent from the other risk factors. In epidemiological studies, this is called the "Finland Factor" (Palace et al., 1999b; Gey et al., 1993). The Lipid Research Clinic Prevention Trial and Follow-up Study showed that high levels of serum carotenoids have a protective role against the incidence of coronary heart disease in a group of patients with the type II hyperlipoproteinemia (Morris et al., 1994; Street et al., 1994). This inverse correlation was found to be significant when compared to the control group; however, this was not found to be true within the group of patients treated with cholestiramine. Cholestiramine is an antihyperlipidemic drug that interferes with the

absorption of carotenoids. The negative correlation between the plasma levels of carotenoids, plasma and the incidence of coronary heart disease was found to be the strongest among the smokers (Morris et al., 1994; Street et al., 1994). The European Multicenter Study has followed 683 subjects with a history of recent myocardial infarction and compared them with 683 matching, healthy controls. The levels of β-carotene stored in adipose tissues were compared with the risk of developing a first myocardial infarction. The results of this study suggested that the normal β-carotene levels were associated with a lower risk of developing an acute myocardial ischemia (Kardinaal et al., 1993).

B.2. Non-supportive studies

Despite these positive findings, several studies have failed to show any beneficial effects of provitamin A carotene intake against the risk of developing cardiovascular disease. Two early, independent studies performed in Finland (1985) and The Netherlands (1987) and later The Cross Cultural MONICA Vitamin Study as well as the large US Physicians Health Study have failed to show any significant association between the higher intake of retinol or low serum β-carotene and the increased risk of coronary artery disease and death from cardiovascular events (Salonen et al., 1985; Kok et al., 1987). The biggest concern was raised with the Beta-Carotene and Retinol Efficacy Trial, where 18 314 smokers, former smokers and asbestos exposed workers were given daily 30 mg of β-carotene and 25,000 IU of retinyl palmitate. These subjects were followed for 4 years. The incidence of lung cancer and cardiovascular disease was examined in the supplemented group and was compared to the matching controls. The authors did not find any evidence of beneficial effect of carotenoids on the incidence of cardiovascular disease. In contrast, they found a significant

increase in cardiovascular mortality in a supplemented group (26 %). The same study also reported an increase in lung cancer mortality in subjects who have taken β-carotene and retinyl palmitate. The study was immediately terminated, 21 months earlier than planned, but a 5 year follow-up was continued (Omenn et al., 1996).

Even though there is an almost equal number of studies that advocate for and against the cardiovascular protective effects of carotenoids, possible mistakes and confounding factors, that are not uncommon in such large studies put their results in question. A lack of proper long-term or short-term sample storage (Gaziano et al., 1992; Hennekens and Gaziano, 1993), non-adjusting results for serum cholesterol levels (Greenberg et al., 1996) and non-accounting, individual diurnal and seasonal variation in vitamin levels (Cantilena et al., 1992) could skew the results and minimize the value of the whole study (Palace et al., 1999b). The new study by Palace et al. (1999a), reported that retinoid plasma levels should not be taken as solemn indicators of retinol status in subjects. This is due to the buffering effect of retinol storage organs in maintaining the constant plasma concentrations, which sheds new light and gives new direction for future research efforts.

B.3. Intervention trials

A number of intervention trials were also performed to show a possible therapeutic and protective usage of vitamin A supplements. The authors of the Indian Experiment of Infarct Survival used large doses of combined vitamin A, C and E supplements in a treatment of 125 patients with diagnosed myocardial infarction (MI). After 28 days of follow-up, the percentage of complications such as arrhythmias and lipid peroxidation products in the serum

of treated patients were lower. Left ventricular function was also found to be improved in the treated group (Singh et al., 1996).

C. Antioxidant Properties of Vitamin A and Carotenoids

C.1. Antioxidant properties of retinol and its metabolites

The antioxidant properties of retinol have been known since the discovery by Monoghan and Schmitt's in 1932. In their study, these authors reported that carotene and vitamin A were able to prevent the oxidation of linoleic acid (Monoghan and Schmitt, 1932; van Jaarsveld et al., 1994). Almost 40 years later, carotenoids' singlet oxygen quenching mechanism was described (Foote and Denny, 1968). However, the biggest break-through was achieved with Burton and Ingold's discovery of carotenoids' lipoperoxide chain-breaking activity that protects against oxidative-induced damage to cellular lipid membranes (Burton and Ingold, 1984).

Retinol's biochemical structure and mobility, due to the short polyene chain, gives it the role of a potent free-radical quenching and peroxidation chain-breaking agent. Retinol can combine with peroxyl free radicals before they can initiate lipid peroxidation (Tesoriere et al., 1993). In this manner, retinol is proven to be a stronger antioxidant than the widely recognized α-tocopherol (vitamin E). However, its lipophilic nature and specific chemical structure limits its antioxidant activities only to lipid environments such as cellular lipid membranes (Palace et al., 1999b). When oxidized by free radicals, retinol forms a chemically labile compound (5,6-retinoid epoxide) that is immediately transformed into a more stable 5,8-oxy derivative. This translocation of unpaired electrons through the conjugated-epoxide, double-bond system protects lipid structures from further peroxidation. The antioxidant

effectiveness of retinol is not only limited to the protection against oxygen-generated free radicals, it is also proven that retinol protects against the potent glutathione radical (D'Aquino et al., 1989).

It is also shown that retinoic acid has antioxidant properties, but the antioxidant potential of this retinol metabolite is diminished by its low physiological concentrations (Samokyszyn and Marnett, 1987). Another retinol metabolite, retinal (retinyl ester) also acts as an antioxidant, however, its antioxidant potential is shown to be much less effective when compared to its parent molecule (Palace et al., 1999b). When an *in vitro* peroxidation system was used to compare the antioxidative potentials of retinol related molecules, retinol was shown to have a much higher antioxidant potential than all of its metabolites. Retinal exhibited a better antioxidant potential than retinyl palmitate, which again was proven to be more potent than retinoic acid (Das, 1989; Palace et al., 1999b).

C.2. Antioxidant properties of provitamin A carotenoids

Provitamin A carotenoids are yellow, red and orange pigmented compounds that were previously described as potent sources of retinol molecules. Even though their nutritional and biochemical significance is still not clear, it is thought that these molecules have an important role in a number of biological processes such as regulation of gap junctions (Bertram, 1993), enhancement of the immune system (Hughes, 1999) and inhibition of mutagenesis, and cell transformation (Krinsky, 1993). However, the most prominent feature of these provitamin A compounds is their potential to act as strong antioxidants *in vivo*. A large number of studies on the antioxidant properties of provitamin A carotenoids have mainly focused on the most abundant and most representative member of this carotenoid family, β-carotene.

Beta-carotene protection against oxidative stress is achieved through its singlet oxygen quenching properties (Rousseau et al., 1992; Burton and Ingold, 1984). Protection by β-carotene is not limited against singlet oxygen only. It has also been shown that β-carotene can act as a chain-breaking antioxidant against lipid peroxidation, which is achieved through its conversion into retinol (Krinsky and Deneke, 1982; Krinsky, 1989). Beta-carotene can also offer protection against the lipid peroxidation of liposomes caused by Fe^{2+}-generated radicals (Krinsky and Deneke, 1982). Through this process, β-carotene prevents the oxidation of lipids and arachidonic acid (Massey and Burton, 1989; Halevy and Sklan, 1987). Beta-carotene can also protect muscle tissue against the free-radical damage caused by lactoperoxidase halide (oxychloride, oxybromide, oxyiodide) (Kanner and Kinsella, 1983), sulfur radicals (Chopra et al., 1993) and sulfonyl and nitrogen dioxide radicals (Everett et al. 1996). This provitamin A can also protect cells against free-radical species that are generated by the enzymatic activity.

C.3. Alternative protection against free-radical induced injury

Retinol and provitamin A carotenoids also exhibit another, alternative, antioxidative protective mechanism. This mechanism is not related to the direct interaction between free radicals and retinoids. This protection is possibly achieved by the initiation of cellular repair processes, through the retinoic acid initiation of DNA transcription. This is achieved through the activation of retinol-specific, intra-nuclear receptors (RXR's and RAR's). Retinol is also known to offer protection to the gap junction protein "conexin." This protein is responsible for the control of cellular division. Conexin damage is thought to be involved in the initiation of uncontrolled cellular proliferation and carcinogenesis (Bates, 1995).

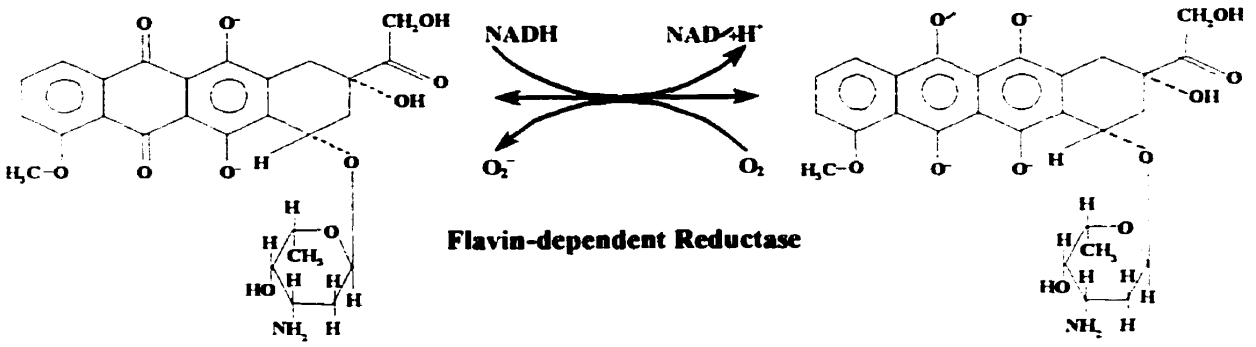
D. Adriamycin

D.1. General facts

Adriamycin is a potent antitumor drug but its use is limited by the development of dose dependent cardiomyopathy. Recent analysis of the literature has revealed that the antitumor and cardiotoxic effects may have two separate mechanisms (Singal et al., 1997). The pathogenesis of adriamycin cardiomyopathy may involve the production of free radicals. These free radicals have a potential to cause damage to the cellular structures. Anthracyclines are known to be powerful inducers of free radicals due to their redox recycling properties (Davies and Doroshow, 1986; Doroshow 1983; Goodman and Hochstein 1977; Olson et al., 1981; Olson and Mushlin, 1990). Free radicals are capable of causing peroxidation of the cellular lipid structures such as mitochondrial membranes and endoplasmic reticulum (Bachur et al., 1978; Thayer, 1977). The fact that the heart has lower antioxidant levels (Doroshow et al., 1979; Revis and Marusic, 1978; Thayer, 1977), and accumulates a high concentration of adriamycin compared to the other tissues, further supports the involvement of adriamycin-free radical injury in the pathogenesis of cardiomyopathy. The antitumor action may be brought about by the intercalation of adriamycin between the DNA base pairs, which causes the inhibition of DNA replication (Sinha and Chignell, 1979; Graves and Krugh, 1983). Adriamycin is also found to bind to the intracellular nuclear enzyme topoisomerase II. This adriamycin-enzyme complex can not repair broken DNA strands and this causes irreparable cellular damage (Graves and Krugh, 1983; Holm et al., 1989). The dual mechanism of adriamycin activity provides a potential for the usage of antioxidants in the prevention of adriamycin cardiomyopathy without interfering with its antitumor activity (Singal et al, 1997).

D.2. Adriamycin-induced free-radical formation and associated damage

A free radical is a molecule or an atom with an unpaired electron in its outermost orbital. This makes it very reactive as an oxidizing and/or reducing agent. Free radicals can initiate a chain reaction leading to production of secondary radicals that can diffuse and cause damage to the distant structures. Adriamycin is a tetracyclic aglycone with a glycosidic bond attached to an amino sugar. The quinone-ring undergoes one electron reduction, by the action of flavin-dependent reductases. This reduction produces a semiquinone intermediate, which is a free radical itself (Figure 3). A semiquinone intermediate can be further reduced into hydroquinone or can be recycled back into quinone with the reduction of one electron. The latter when captured by oxygen, results in the production of a superoxide radical (Davies and Doroshow, 1986; Basser and Green, 1993). This superoxide radical, through the enzymatic activity of superoxide dismutase, donates an electron to a large number of compounds to yield molecular oxygen and hydrogen peroxide (H_2O_2). Hydrogen peroxide can be further reduced to produce a hydroxyl radical. These free radicals can initiate lipid peroxidation (Mimnaugh et al., 1981; 1985). It is also possible that adriamycin can directly cause the reduction of Fe^{3+} to Fe^{2+}, a free radical that can initiate further oxidative damage (Hasinoff and Davey, 1988; Minotti and Aust, 1987). Adriamycin-Fe^{3+} complex can also initiate lipid peroxidation without the involvement of oxygen free radicals (Gutteridge, 1983; Sugioka and Nakano, 1982). This complex has a high affinity for binding and damaging cardiolipin, an important mitochondrial and sarcoplasmic membrane phospholipid (Hasinoff and Davey, 1988; Demant, 1984). This phospholipid is responsible for the activity of a large number of membrane-bound enzymes such as mitochondrial cytochrome-c oxidase



ADRIAMYCIN (QUINONE) ADRIAMYCIN (SEMIQUINONE)

Figure 3: Mechanism of adriamycin's cycling between quinone and semiquinone form.

(respiratory enzyme) (Hasinoff and Davey, 1988; Davies and Doroshow, 1986). The important role of iron in adriamycin cardiotoxicity is proven by the findings that ICRF-187, an iron binding chelator, can prevent the development of cardiomyopathy (Herman et al., 1981). Lower levels of antioxidant enzymes (Odom et al., 1992), a high content of cardiolipin and a large number of mitochondria due to high metabolic and respiratory activity make the heart more susceptible to this drug-induced toxicity than other organs.

D.3. Cytotoxicity

Adriamycin-mediated, cytotoxic antitumor action has been explained by several different mechanisms including free-radical induced strand breaks (Bachur et al., 1992), inhibition of mitochondrial oxidative phosphorylation (Muhammed et al., 1982), adriamycin-Fe^{3+} complex covalent binding of to DNA (Keizer et al., 1990; Myers et al., 1986), membrane modulation (Tritton, 1991), intercalation between DNA base pairs (Bodley et al., 1989) and inhibition of topoisomerase I and II (Tewey et al., 1984). Adriamycin binding to DNA results in conformational changes of DNA structure (stiffening, bending and

elongation) (Patel et al., 1981) and loss of adriamycin free-radical producing potential (Berg et al., 1982). It is believed that iron plays a crucial role in the cytotoxic effect of adriamycin. This adriamycin-Fe^{3+} complex (Eliot et al., 1984) not only disrupts normal protein production, but also allows adriamycin to keep its free-radical generating potential, so it is able to catalyze the production of free radicals in the close vicinity of DNA resulting in extensive DNA cleavage (Eliot et al., 1984; Muindi et al., 1985). Recent studies are more focused on topoisomerase II as a major target for the whole anthracycline family. In the G2 interval of the mitotic process, the drug-DNA-topoisomerase complex interferes with the movement of the replication fork leading to irreparable cellular damage (Holm et al., 1989). It is thought that the cells with the highest levels of topoisomerase II are found to be most aggressive and have higher proliferative potential (Holden et al., 1990). The study performed on the cells with the high concentration of topoisomerase II (CHO mutant ADR-1 cells) showed that these cells were also found to be the most susceptible to adriamycin cytotoxic action (Davies et al., 1988). Free-radical induced damage of DNA has been proven only in *in vitro* studies using extremely high drug concentrations.

D.4. Adriamycin-induced cardiotoxic effects

Based on the times of their onset, all of the cardiotoxic effect can be divided in to three groups: acute, subacute and chronic. The acute and subacute cardiotoxic effects of adriamycin are preventable and/or clinically manageable. However, the most serious toxic side effect of adriamycin's chronic administration is the development of dose-dependent, drug-induced, cardiomyopathy (Buja et al., 1973; Lefrak et al., 1973; Von Hoff et al., 1979).

D.4.a. Chronic cardiotoxicity (adriamycin-induced cardiomyopathy). This toxicity was reported in a study of the charts of 339 cancer patients treated with adriamycin (Lefrak et al., 1973). An association between adriamycin administration and development of drug-induced cardiomyopathy in doses higher than 550 mg/m^2 of body surface area was discovered. It was also reported that adriamycin-induced cardiomyopathy could eventually lead to the development of congestive heart failure (CHF) (Lefrak et al., 1973). Reports on dose-dependent cardiotoxicity eventually resulted in the limitation of cumulative doses of adriamycin below 550 mg/m^2 of body surface area. This limitation has led to the decrease in the initial incidence of 4-10% of patients that would eventually progress to adriamycin-induced cardiomyopathy and heart failure. The major characteristic of adriamycin-induced heart failure is its refractoriness against inotropic and mechanical assistance therapy (Lefrak et al., 1973). Once established, the prognosis for these patients is very poor (Bristow et al., 1978; Lenaz and Page 1976; Von Hoff et al., 1979; Minow et al., 1977).

D.4.a.i. Major pathological and functional changes. Gross pathological features of this heart failure, in its end stage, are generalized cardiomyopathy, development of mural thrombi, multifocal area of interstitial fibrosis and myocardial degeneration (Buja et al., 1973). Functional changes in this pathological state include hypotension, tachycardia, ventricle dilatation, decreased ejection fraction and depression of QRS complex (Lefrak et al., 1973; Singal et al., 1987).

D.4.a.ii. Histopathological changes. Two main types of adriamycin-induced histopathological changes were reported in humans (Billingham et al., 1977; Bristow et al.,

1978; Buja et al., 1973), rabbits (Bachur et al., 1974; Jaenke, 1976), mice (Rosenoff et al., 1975; Myers et al., 1977) and rat models (Singal et al., 1985).

The first type of histopathological damage is more prevalent in humans and is characterized by the partial and, in the later stage, total loss of myocytes. Even though myocytes are lost due to adriamycin toxic effects, the nuclei, Z lines and mitochondria of adriamycin-treated cells appear unaffected. The second type of damage is represented by a distension of the sarcotubular system that causes the vacuolar degeneration of myocytes. It is presumed that this swelling is caused by the adriamycin-induced peroxydative damage to the membranes of the sarcoplasmic reticulum (Singal et al., 1987).

Other structural abnormalities include: mitochondrial damage, lipid accumulation and an increased number of lysosomes (Singal et al., 1985; Ferrans, 1978).These morphological changes are evaluated by a grading system which can be applied to myocardial biopsy specimens to evaluate and manage the dosage of adriamycin in a treatment regime (Bristow et al., 1978; Friedman et al., 1978).

D.4.a.iii. Subcellular changes. A large scale of adriamycin-induced functional changes on the cardiomyocyte level were also reported. Adriamycin is known to stimulate Ca^{2+} ATPase activity (Singal and Pierce, 1986). This enzyme plays an important role in the regulation of low-affinity Ca^{2+} binding, which effects the myocardial contractile force (Dhalla et al., 1982; Bers et al., 1981). Adriamycin is also shown to stimulate voltage-dependant, slow Ca^{2+} channels (Azuma et al., 1981). Through this mechanism, adriamycin increases the cell membrane permeability for Ca^{2+} ions which can cause intracellular Ca^{2+} overload (Kusuoka et al., 1991). The increased permeability of cellular membranes is supported by

clinical findings which show that patients who receive adriamycin exhibit an increase in serum LDH, CPK and SGOT (Lefrak et al., 1973). The increase in serum levels of these intracellular enzymes can be explained by their leakage through the adriamycin-damaged cellular membranes. Adriamycin-induced damage to the sarcoplasmic reticulum (SR) can seriously impair the contraction-relaxation coupling process in the heart muscle, which can lead to changes in its contractility. Mitochondrial changes are characterized by swelling and the presence of intramitochondrial amorphous dense bodies (Singal and Pierce, 1986). These pathological changes, caused by the intramitochondrial accumulation of Ca^{2+} (Miwa et al., 1986), can disrupt ATP-energy production through the Ca^{2+}-induced inhibition of enzymes involved in oxidative phosphorylation (Dhalla et al., 1982; Singal et al., 1985). Adriamycin is also shown to decrease the concentration of cellular high-energy phosphates (Azuma et al., 1981; Pelikan et al., 1986; Singal and Pierce 1986). Lysosomal changes include an increase in the number of lysosomes, lysosomal leakage and consequent damage to cardiomyocytes (Singal et al., 1985), which can be attributed to the previously explained adriamycin-induced free radical and lipid peroxidative damage to lipid membranes.

D.4.a.iv. Risk factors. Several risk factors are also known to contribute to these toxic effects. They include old age (Von Hoff et al., 1977; 1979; Bristow, 1979; Weinberg and Singal, 1987), mediastinal radiation therapy (Billingham et al., 1977; Gilladoga et al., 1976; Minow et al., 1977), liver dysfunction (Bachur, 1979; Benjamin, 1975), combination chemotherapy (Praga et al., 1979; Minow et al., 1977), a history of previous cardiac diseases (Bristow et al., 1980; Singal et al., 1987), and hyperthermia (Kim et al., 1979).

D.5. Mechanisms of protection:

Finding the mechanism that would offer adequate protection against adriamycin-induced cardiomyopathy remains a challenge. This is mainly due to the fact that the pathogenesis of this drug-induced toxic effect still remains unclear. The main prerequisite for the future protective agents is that they have to offer adequate protection against cardiovascular effects without interfering with adriamycin's antitumor effect. Several conceptually different approaches have been followed to minimize the cardiotoxicity of the less toxic analogues; and concomitant administration with antioxidants and other agents. The following discussion is limited to the use of antioxidants and other agents:

D.5.a. Antioxidant protection. As previously described, adriamycin is a potent free-radical generator which can cause a disruption in the balance between free-radical production and endogenous antioxidant protection mechanisms, leading to the state known as oxidative stress (Singal and Kirshenbaum, 1990; Kalyanaraman et al., 1980). Several antioxidants have been used in an attempt to prevent the development of adriamycin-induced cardiomyopathy.

D.5.a.i. Nonenzymatic antioxidant protection. Naturally occurring, nonenzymatic antioxidants have been used to prevent adriamycin-induced oxidative stress. A large number of studies were specifically focused on the usage of vitamin E, the most potent biological antioxidant vitamin. Rats fed with a vitamin E deficient diet have expressed significantly higher mortality and deteriorating ultrastructural damage due to the administration of adriamycin when compared with animals fed a normal diet (Singal and Tong, 1988). The study performed by Myers and his colleagues using the mouse model showed that the vitamin E pretreatment prevented the development of adriamycin-induced ultrastructural changes in

the hearts of adriamycin-treated animals (Myers et al., 1977).Another study showed that vitamin E administration prevents only the acute toxic effects of adriamycin, but it does not alter the development of chronic cardiomyopathic changes (Mimnaugh et al. 1979).A large number of studies have questioned the protective role of vitamin E against adriamycin-induced oxidative stress (Klein, 1995). Its combined usage with selenium did not provide any significant protection against adriamycin's toxic effects in heart and liver tissues (Hermansen and Wassermann, 1986), while selenium administration, itself, was shown to protect against chronic cardiotoxicity (Boucher et al., 1995). Another nonenzymatic antioxidant, ascorbic acid (Vitamin C), was also shown to protect against adriamycin-induced oxidative stress by regenerating reduced forms of vitamin E and making them available for protection against membrane lipid peroxidation. Vitamin E acts against lipid peroxidation in cell membranes, while vitamin C offers effective protection against free-radical damage to the cytosolic structures (Kaul et al., 1993). However, the exact mechanisms of vitamin antioxidant protection still remain to be fully examined.

D.5.a.ii. The usage of vitamin A against adriamycin-induced cardiomyopathy. Vitamin A (25 IU/kg of body weight), administered two days prior to adriamycin treatment (single injection 10 mg/kg i.p.) was able to substantially reduce adriamycin-induced peroxidative damage both to the heart lipids and proteins in the heart and significantly lowered the serum values of lactate dehydrogenase and creatine phosphokinase (Tesoriere et al., 1994). Vitamin A pretreatmnet also prevented histopathological changes induced by adriamycin administration and significantly increased the survival rate of the animals (Tesoriere et al., 1994). Contradictory results were obtained in the 1985 study performed by

Nakagawa et al. Using a P388 leukemia mouse bearing model, these authors showed that retinol administration can potentiate the antitumor action of several different antineoplastic drugs including adriamycin. The exact mechanism of this antiproliferative action of retinol still remains to be explained (Nakagawa et al., 1985). However, it is thought that this protective mechanism is mediated by the retinol metabolite, retinoic acid, that is involved in the regulation of cell differentiation. It can be concluded that previous studies have yielded promising results on the possible protective role of retinol in the prevention and treatment of adriamycin-induced toxic effects, of which adriamycin cardiomyopathy is the most serious one. The prevalent problem of toxic vitamin A hypervitaminosis should be avoided by using a smaller concentration of retinoids. However, more studies must be performed, especially clinical studies and a lot of persistent questions have to be answered before an exact conclusion may be drawn.

D.5.a.iii. Enzymatic antioxidants. Studies using transgenic mouse models that overexpress the antioxidant enzymes catalase and superoxide dismutase showed a significant decrease in the severity of adriamycin-induced myocardial damage (Kang et al., 1996; Yen et al., 1996).

D.5.b. Use of other agents against adriamycin induced cardiomyopathy.

D.5.b.i. Iron chelation. Due to the important role of iron in adriamycin-induced free radical production, several iron chelators were used in order to prevent the development of adriamycin induced cardiotoxic effects. ICRF-187 (dextrazoxane) was shown to protect against the damage from semiquinone radicals by chelating its co-factor, iron. The study performed on female breast cancer patients reported that dextrazoxane was able to prevent

the development of adriamycin-induced oxidative damage to the myocardium (Speyer et al., 1988; 1992; Seifert et al., 1994). The usage of ICRF-187 was also proven to be effective in preventing adriamycin-induced cardiomyopathy in pediatric cancer patients (Bu'Lock et al., 1993). Currently, dextrazoxane is only approved for usage in female breast cancer patients who are receiving anthracycline treatment in cumulative doses that exceed 300 mg/m^2 of body surface area (Shan et al., 1996), even though the adriamycin-induced cardiotoxic effect is reported to develop in lower cumulative doses. The concern with ICRF-187 usage is that its combination with anthracyclines is associated with increased hematological toxicity (Von Hoff et al., 1981) and decreased antitumor effect of adriamycin (Sehested et al., 1993). It is also reported that dextrazoxane can initiate the progression of an early stage breast cancer (Seifert et al., 1994).

D.5.b.ii. Free-radical scavengers. Oleanolic, urosolic and selenoroganic compounds, all free-radical scavengers, were shown to be potent protectors of heart and liver tissues against adriamycin-induced peroxidation *in vitro* (Balanehru and Nagarajan, 1992; Pritsos et al., 1992). The use of ambroxol, flavonids, N-acetyl cysteine and cahectin was also examined; however, the protection offered by these agents was found to be adequate only in *in vitro* systems (Myers et al., 1977; 1983; Doroshow et al., 1981; Unverferth et al., 1983; Herman et al., 1985; Kozluca et al., 1996; Husken et al., 1995; van Acker et al., 1995).

D.5.b.iii. Melatonin. Recent data, presented by Morishima et al. (1998), have shown that melatonin, a pineal hormone with antioxidant properties, was also able to provide adequate protection against adriamycin-induced cardiomyopathy in a rat model. Further studies should be performed to explain the protective effect of this hormone.

D.5.b.iv. Protection using probucol. The most promising results in protection against adriamycin-induced cardiomyopathy were achieved with the administration of probucol, a well-established, anti-hyperlipidemic drug with antioxidant properties (Siveski-Iliskovic et al., 1994; 1995). Pre- and concurrent treatment with 20 mg/kg of probucol completely prevented development of the acute and chronic toxic side effects of adriamycin (Singal et al., 1995), without interfering with its antitumor effects (Siveski-Iliskovic et, al. 1995). Probucol acts as a potent free-radical scavenger as well as a promoter of endogenous myocardial antioxidant enzymes (Siveski-Iliskovic et al., 1994). It can reduce the peroxidation of membrane lipids, thus minimizing the cardiotoxic effects produced by adriamycin treatment (Singal and Iliskovic, 1998). The potential usage of probucol in a clinical setting still remains to be examined.

E. Vitamin E

E.1. Brief history and chemical properties

In 1922, Evans and Bishop were the first ones to report on a "compound x" which they found present in lettuce. Because of its structural and biochemical characteristics, the newly found compound was included in the large, growing family of vitamins and was later named vitamin E by Evans (Machlin et al. 1985). Although the vitamin E discovery was made several decades ago, its full physiological role still remains to be discovered.

E.2. Chemical structure

As previously said, the vitamin E family consists of two subgroups of four naturally-occurring compounds; tocopherols and tocotrienols. Tocopherols and tocotrienols are, by their chemical structure, isoprenoid-substituted 6-hydroxychromanes or tocols. The main

difference between these two subgroups is that tocopherols have saturated, while tocotrienols have unsaturated, lipophilic side chain. Alpha-tocopherol and α-tocotrienol both have three methyl groups added on 5', 7', 8' positions. Beta- and γ-tocopherols and tocotrienols have two methyl groups in 5', 8' and 7' and 8' positions respectfully. Delta-tocopherol and tocotrienol have only one methyl group in the eight position of their side chain (Figure 4).

The RRR-α-tocopherol stereoisomer has the widest natural distribution and highest biological activity. It is estimated that about 80% of the total vitamin E activity is attributable to α-tocopherol and most of the remaining to γ-tocopherol (Bieri and Evarts, 1973). The biological activity of vitamin E members is expressed in international units or D-α-tocopherol equivalents. The activity of 1 mg of 2',4' and 8' synthetic isomeric carbon all-rac-α-tocopherol is considered to be equal to 1 international unit.

Daily requirements for α-tocopherol are 2-2,5 mg/day in neonates and up to 6-10 mg/day in adults. These daily requirements increase with an increase in the dietary intake of polyunsaturated fatty acids.

E.2. Antioxidant properties of vitamin E.

A large number of enzymes and enzymatic systems with the potential for free radical production, are incorporated in biomembranes which makes them a very preferable target for free-radical attack. Free radicals produced by these enzymatic systems are able to induce chain lipid peroxidation. Lipid peroxidation can interfere with normal membrane function, signal transduction through the membranes and can result in membrane cluster formations, which interfere with the membrane semi-permeability characteristic (Meerson et al., 1982). However, the specific position of vitamin E in cellular membranes provides adequate

protection against various pathological processes. Compared to the other tocopherols, α-tocopherol exhibits the highest biological and antioxidative properties (Century and Horwitt, 1965).

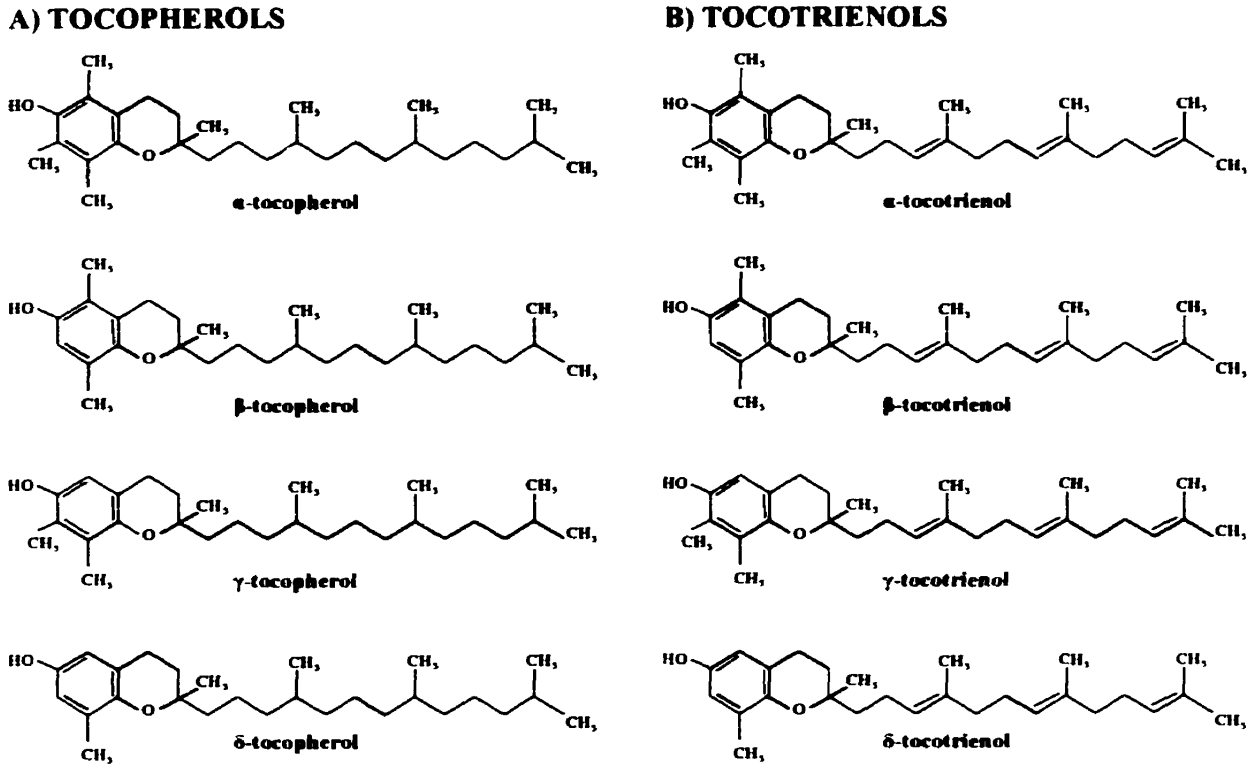


Figure 4: Naturally occurring forms of vitamin E.

IV. MATERIALS AND METHODS

A. Animal Model and Treatment Protocol

Male Sprague-Dawley rats (body weight, 320 ± 10 g) were housed in pairs and maintained on commercial rat chow (PMI Feeds, MO, USA), and water *ad libitum*. All the animals used in the study were maintained and treated in accordance to the policies and procedures of Canadian Council of Animal Care (CCAC). Animals were divided into two groups: control untreated (CONT) and adriamycin treated (ADR). In the ADR group, Adriamycin (doxorubicin hydrochloride) was administered in 6 equal injections (2.5 mg/kg ADR each) over a period of 2 weeks for a cumulative dose of 15 mg/kg body weight (Siveski-Iliskovic et al., 1994; 1995). Animals observed for 21 days after the last injection, were assessed for hemodynamic function and sacrificed for further studies on the heart, plasma, liver and kidneys.

B. Study of hemodynamic function and ascites

Animals were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally). Left ventricular systolic and diastolic pressures, as well as aortic peak systolic and diastolic pressures were assessed by introduction of a miniature pressure transducer (Millar-Micro-Tip) through the right carotid artery into the aorta and left ventricle (Hill and Singal, 1997). The data were recorded for on-line analysis using an Axotape acquisition data program. Fluid from the peritoneal cavity was collected in a measuring cylinder for quantitating ascites.

C. Collection of Tissue and Plasma

Blood was collected by cardiac puncture with a 20-gauge needle in a 5 c.c. heparinized syringe. The samples were immediately centrifuged at 4,500 RPM for 15 minutes to separate the plasma, which was then frozen in liquid nitrogen and kept in light-protected vials. The liver, kidney and heart were immediately removed, weighed, frozen in liquid nitrogen and stored in the light-protected containers until analysis.

D. Plasma Retinol and α-tocopherol Measurements

Two-hundred (200) µl of plasma was pipetted into a microcentrifuge tube and an equal volume of HPLC-grade ethanol containing tocopherol acetate as internal standard was added to correct for recovery in each sample. The solution was briefly mixed, and 500 µl of 3:2 HPLC grade Ethyl acetate: hexane was added for the extraction. The solution was incubated for 5 minutes, protected from light. The mixture was then centrifuged at 10,000 RPM for 2 minutes. Two-hundred and fifty (250) µl of top layer was retrieved, dried, resuspended in 125 µl of the mobile phase and analyzed in HPLC. Retention peaks obtained at 4.7 and 11.6 min. for retinol and α-tocopherol were read. Data were expressed in µg/ml of plasma.

E. Retinol and α-tocopherol Measurements in the Heart

Small portion of the heart tissue (0.1-0.3 g) was suspended in 4 ml of double-distilled water (DDW) and homogenized using Polytron Homogeniser (Kinematica Instruments, Switzerland). Two (2) ml of HPLC-grade ethanol with internal standard was added and briefly vortexed (Fisher, Bohemia, NY, USA). For the extraction, 500 µl of 3:2 HPLC grade Ethyl acetate: hexane was added, mixed, and incubated at room temperature for 5 minutes

in vials protected from light. These tubes were centrifuged at 4,000 RPM for 10 minutes using a table top centrifuge (Sorvall instruments, CT, USA), and 1.5 ml of the top layer was retrieved. This supernatant was dried completely in a vacuum using a Speed Vack evaporator (Savant Instruments, NY, USA). The vials were protected from light and frozen in liquid nitrogen and stored at -79°C until analyzed. The samples were resuspended in 125 µl of the mobile phase and analyzed using Beckman HPLC systems. Data were expressed in µg/g of tissue.

F. Liver and Kidney Retinol and α-tocopherol

Kidney and liver tissue, 0.1 g, each was homogenized in 2 ml of DDW using Polytron Homogenizer (Kinematica Instruments, Switzerland). Later, the sample was analyzed using the same preparation and extraction procedures previously described for plasma (Palace et al., 1994). Data were expressed in µg/g of tissue.

G. Preparation of ^{3}H-retinol and its administration

Seventeen days after the last injection of adriamycin, animals were anesthetized with sodium pentobarbital (50-mg/kg i.p.). Reconstituted retinol (50 µl) containing ^{3}H-retinol, suspended in plasma, was injected through a previously placed catheter into the tail vein of each animal which was followed by an injection of a small volume (200 µl) of physiological saline. The ^{3}H retinol suspension was prepared from plasma that was previously obtained from rats of the same age and strain. The plasma had been striped from its native retinol but not the retinol binding protein, which was achieved by overnight incubation with the activated charcoal (Green et al., 1993). This method removes for more than 98% of the native retinol (Palace et al., 1994). The injected ^{3}H-retinol contained 0.00334 µg of retinol bound to

retinol-binding protein (RBP) with total activity of 5 μCi. This amount of retinol represents less than 1% of the total plasma retinol in rats (Harrison and Gad, 1989). Between the injections, the cannula was refilled with 250 I.U.of heparin and 1 ml of physiological saline and its end was sealed.

H. ^{3}H Activity Measurements in Tissues and Plasma

The amount of ^{3}H activity was detected by manual fraction collection at specific peak retention times (5-6.2 minutes). Fractions were suspended in 8 ml of Ecolume scintillation liquid (ICN Radiochemicals, Irvine, CA) and counted for 10 minutes on a Beckman scintillation counter (Beckman Instruments, Irvine, CA). Scintillation liquid was used as the reference value for the background radiation. Data are expressed in pg/ml for the plasma and pg/μg of retinol for tissues.

I. Retinol Ester Hydrolases

I.1. Bile Salt-independent (BSI)

A known quantity of the liver was homogenized in 60 ml of 50 mM Tris-maleate buffer in saline. Three aliquots were saved in dark, light-protected vials. Ten (10) ml of homogenate was kept on ice in dark microcentrifuge vials and later centrifuged at 15000 RPM for 20 minutes using a J2-Hs Beckman microcentrifuge (Beckman Instruments, Ca, USA) with a JA20 rotor. Five (5) ml of clear supernatant was pipetted into microcentrifuge vials, weight-balanced and again centrifuged at high speed (37000 RPM) using Beckman L7-56 ultracentrifuge (Beckman Instruments, CA, USA) with Ti 70 rotor for 75 minutes at 4°C. Supernatant was discarded and a white-brown colored pellet, containing microsomes,

was resusepened in 50 mM tris-maleate buffer and vortexed for 2 minutes. Two (2) aliquots of 1 ml each were immediately frozen in liquid nitrogen and kept at -79°C.

Retinol palmitate (690 mg) was dissolved in 10.5 ml of hexane. This was mixed and dried completely using a stream of gaseous nitrogen. Then, 2.5 ml of assay buffer was added which was made up of 200 ml of triton-x100 dissolved in 10 ml of Tris maleate buffer (pH=7.0). Microsome fraction (15 µl, containing 300 µg of protein) was mixed with 10 µl of retinol palmitate solution and incubated for 45 minutes in a water bath at 37°C.. The reaction was stopped by adding ethanol, and retinol was extracted using 0.5 ml of ethanol/hexane. Blank samples had ethanol added before the incubation in order to stop further reaction (Palace et al., 1994). After the extraction, samples were centrifuged at 10,000 RPM using a J2-Hs Beckman microcentrifuge (Beckman Instruments, USA), and 250 µl of the top layer was recovered and dried using Speed-Vack evaporator (Savant Instruments, NY, USA) under the vacuum for 45 minutes. Dry samples were stored at -79°C and resuspended, in 125 µl of the mobile phase, which was then injected into HPLC system. Results are expressed as pmol/min/mg of protein.

I.2. Bile Salt-dependent (BSD)

Chaps buffer (350 µl) was mixed with 50 µl of retinol palmitate and this was pre-incubated for 5 minutes in a water bath at 37°C. Proteins were estimated using the method by Lowry (Lowry et al., 1951). A volume of liver homogenate containing 500 µg of protein was added into the vials (in this case it was 10 µl). The mixture was incubated at 37°C in a water bath for 60 minutes. Ethanol (500 µl) was added to the samples and mixed. The mixture was then centrifuged at 10,000 RPM using J2-Hs Beckman microcentrifuge

(Beckman Instruments, CA, USA). Two-hundred and fifty (250) µl of the top layer was recovered and then dried in a vacuum using a Speed-Vack evaporator (Savant Instruments, NY, USA) for 45 minutes and stored at -79°C until the samples were analyzed. Samples were later re-suspended in 125 µl of the mobile phase and injected into HPLC. The data are represented as pmol/min/mg of protein.

K. HPLC Technique

Retinol and α-Tocopherol levels were measured in plasma, heart, liver and kidney using the normal phase HPLC method (Palace et al., 1994). A silica HS C18, 5 µm pore size column (250 x 4.6 mm) was used. A mobile phase of 70:20:10 (v/v/v) acetonitrile:diclomethane:methanol delivered at 1 ml/min was used to extract the samples. All reagents of HPLC standards were obtained from Sigma. The samples were injected into HPLC through a 100 µl loop using a light-protected syringe. Retinol and retinol esters were detected and eluded at 325 nm (up to 7 minutes) and α-tocopherol and tocopherol acetate at 292 nm (up to 30 minutes) using a Beckman 166 detector (Palace et al., 1994). The total run time was 35 minutes. The typical retention times for retinol, α-tocopherol, tocopherol acetate and retinol palmitate were detrmined. The standard curve for retinol was determined by injecting a standard solution of retinol (100 mg/100 ml of retinol:ethanol) in three concentrations 100 ng, 50 ng and 25 ng with peak areas 8.438, 4.429 and 2.1019. The standard curve showed good linearity.

L. Statistical Data Analysis

Data are expressed as the mean ± SEM with the number of animals noted in the tables. For statistical analysis of the data, group means were compared by one-way analysis of

variance and the data from the CONT and ADR groups were compared using the student t-test. Statistical significance was acceptable for levels of $P<0.05$.

V. RESULTS

A. General Observations

The general appearance of the animals in the control (CONT) and adriamycin (ADR) groups was observed during the course of the study. Adriamycin-treated animals showed development of a scruffy fur with a light yellow coloration. Animals appeared sick, weak and lethargic. The CONT group of animals did not show any changes in their general appearance. The most prominent feature in the adriamycin-treated animals was the development of dyspnea, presence of a grossly enlarged abdomen and abdominal fluid (transudate). This pathological condition became evident in the first week after the completion of adriamycin administration and worsened with time. The amount of peritoneal fluid at the end of 3 week post-treatment in the ADR group was 63.6 ± 10.1 ml while there was no ascites in the CONT animals (Table 1).

A significant decrease in the body weight in the ADR group was noted soon after the beginning of ADR treatment. There was a slight improvement in the body weight during the post treatment period. However, at the end of 3 weeks of post-treatment period, ADR-group animals still had a lower body weight as compared to CONT group. The rats in the ADR group showed a significant decrease in heart weight (HW) compared to animals in the CONT group (Table 1). Heart weight/body weight (HW/BW) ratio was also decreased in the ADR group as compared to the control, but this difference was not found to be significant. These pathological changes are consistent with the development of classical heart failure features due to adriamycin treatment.

Table 1: Hemodynamics, ascites and heart weight in adriamycin-induced cardiomyopathy.

PARAMETER	CONTROL	ADRIAMYCIN
LVSP (mmHg)	122.2 ± 10.2 (6)	91.1 ± 5.3* (6)
LVEDP (mmHg)	5.8 ± 2.6 (6)	29.6 ± 3.2* (6)
ASP (mmHg)	116.6 ± 6.9 (6)	89.5 ± 4.6* (6)
ADP (mmHg)	71.8 ± 7.2 (6)	69.4 ± 3.9 (6)
Ascites (ml)	0 (6)	63.6 ± 10.1 (9)
HW (g)	1.53 ± 0.05 (6)	1.18 ± 0.03* (9)
HW/BW	3.20 ± 0.09 (6)	2.99 ± 0.11 (9)

Data are mean ± S.E. *) Significantly different ($p<0.05$) from the control value. LVSP, left ventricular systolic pressure; LVEDP, left ventricular end diastolic pressure; ASP, aortic systolic pressure; ADP, aortic diastolic pressure; HW, heart weight; and BW, body weight. The number in parenthesis indicates the number of animals.

B. Hemodynamic Studies

At the end of 3-week post-treatment period, animals were assessed for their hemodynamic function. Adriamycin-treated rats exhibited about 410% increase in left ventricular end-diastolic pressure (LVEDP) compared to the control and the change was statistically ($P<0.05$) significant. The left ventricular systolic pressure (LVSP) and aortic systolic pressure (ASP) were significantly decreased by about 25% and 23%, respectively, in the ADR group compared to the CONT group. Aortic diastolic pressure (ADP) in the ADR group was unchanged (Table 1).

C. Retinol in the Heart and Plasma

Retinol levels in the plasma of control animals were 0.30 ± 0.19 μg/ml and these levels were not changed in the plasma collected from the ADR group (Fig. 5). In the hearts of CONT group animals, retinol levels were 0.277 ● 0.444 μg/g . Although there was a trend towards a decrease in the retinol levels in the ADR group compared to CONT, this change did not reach acceptable levels of significance (Fig.5).

Radio labeled retinol levels in the plasma of CONT group animals were 1.943 ± 0.25 pg/ml. These levels were found to be increased by about 76 % in the ADR group and this change was significant ($p<0.05$). Radio labeled retinol levels in hearts of CONT group animals were 1.526 ± 0.08 pg/μg and these levels were found to be increased by about 81% in the ADR group (Fig.6).

D. Liver and Kidney

In the liver, retinol levels in CONT group were 8.063 ● 0.72 μg/g and these levels in the ADR group were decreased by about 47%. Retinol is stored in the tissues in the form

of retinol esters such as retinol palmitate. Thus the levels of retinol palmitate were also analyzed. In the CONT group, the level of retinol palmitate in the liver was 103.22 ± 6.03 μg/g and these levels were decreased in ADR group by about 39 %(Fig. 7).

Radio labeled retinol levels in the liver and kidneys in the CONT group was 1.89 ± 0.12 pg/μg and 1.52 ± 0.22 μg/g of retinol .In the ADR group these levels were found to be increased by 103% and 78% respectively (Fig.8).

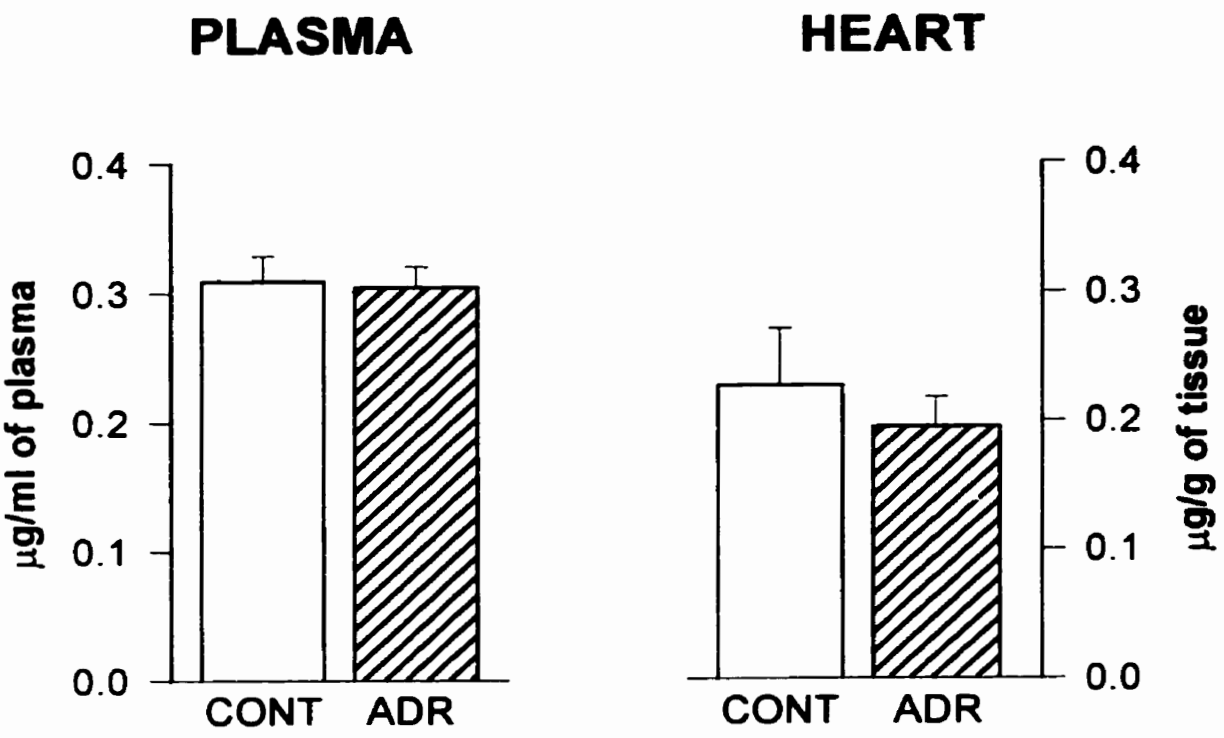


Figure 5: Retinol in the plasma and hearts from Control (CONT) and Adriamycin (ADR) groups. Data are expressed as mean ± SEM of 6 animals in CONT group and 9 in ADR group.

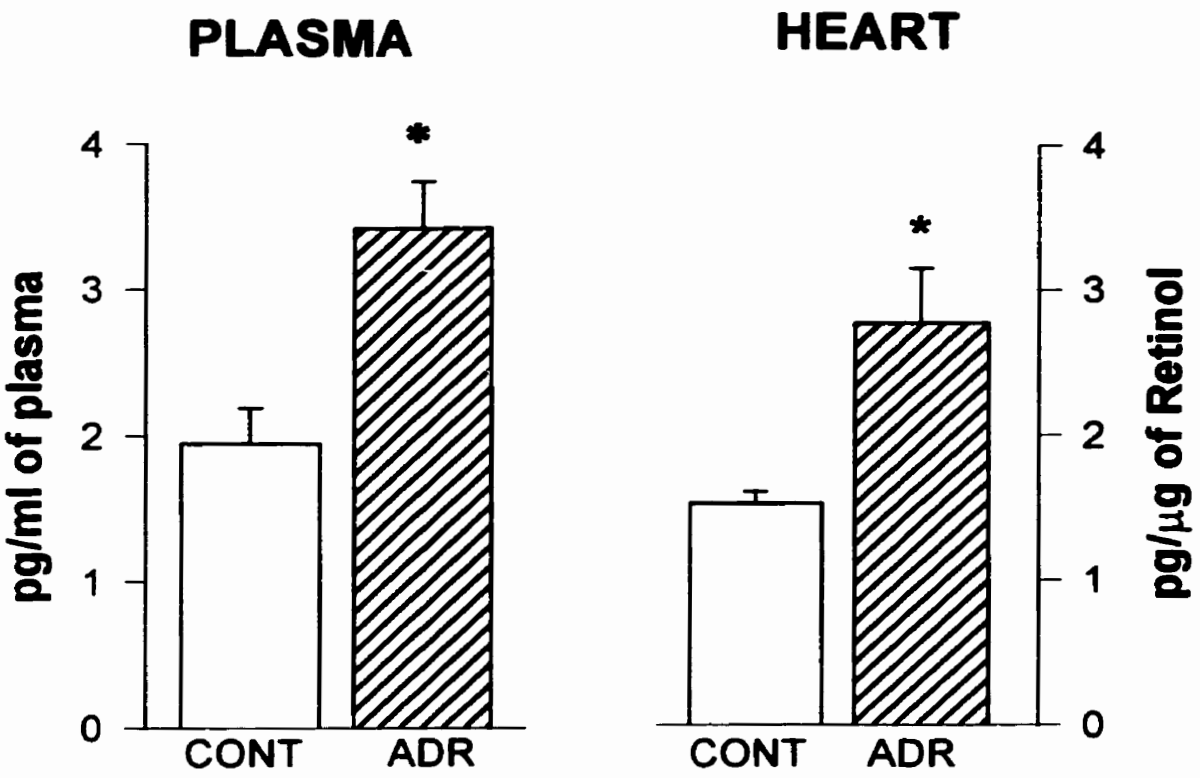


Figure 6: ^{3}H radiolabeled retinol levels in heart and plasma of Control (CONT) and Adriamycin-treated (ADR) groups. Data are expressed as mean ± SEM of 6 animals in CONT group and 9 in ADR group. *) represents significant difference ($p<0.05$).

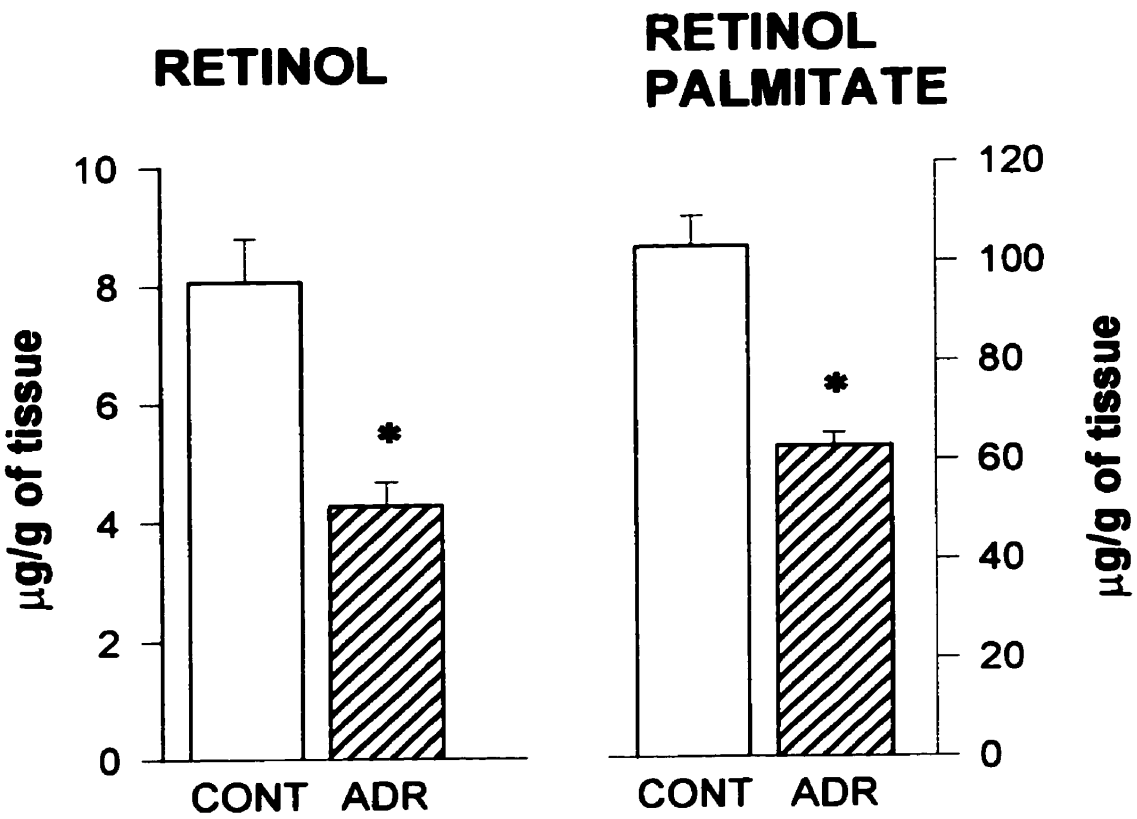


Figure 7: Total retinol and retinol palmitate levels in the liver from Control (CONT) and Adriamycin (ADR)groups. Data are expressed as mean ± SEM of 6 animals in CONT group and 9 in ADR group.*) represents significant difference ($p<0.05$).

In the kidneys retinol and retinol palmitate levels were 2.15 ± 0.16 and 8.03 ± 0.30 μg/g respectively. There was no significant difference between ADR and CONT in their retinol and retinol palmitate levels (Fig.9).

E. REH Activity

Retinol is released from the retinol esters by retinol ester hydrolase enzymes. Bile-salt dependant (BSD) and bile-salt independent (BSI) retinol ester hydrolase activities in the CONT group were 126.56 ± 26.06 pmol/min/mg and 9.47 ± 0.56 pmol/min/mg of protein (Fig. 10). Both activities were decreased in ADR group ($p<0.05$).

F. Alpha-Tocopherol Levels in the plasma and tissues

Alpha-tocopherol levels in the plasma of CONT animals were 5.95 ± 0.43 μg/ml and these levels were found to be increased by about 109% in the ADR group (Fig.11). The liver α-tocopherol levels in the CONT group were 35.35 ± 2.73 μg/g and these levels were significantly ($P<0.05$) increased in ADR group by about 29%(Fig.12). Alpha-tocopherol levels in the kidneys in the CONT group were 15.67 ± 1.71 μg/g and these levels were found to be increased by 30% in the ADR group. Heart did not show any significant change in α-tocopherol levels after the administration of adriamycin and the values in CONT group were 39.79 ± 1.90 (Fig.12).

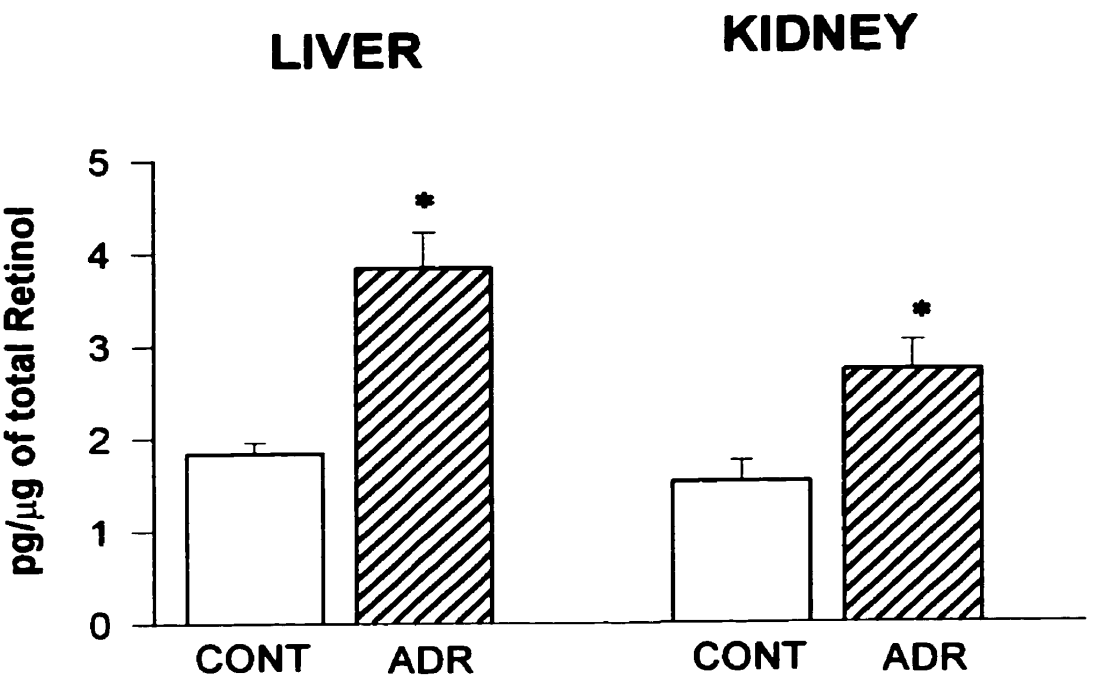


Figure 8: ^{3}H radiolabeled retinol levels in kidneys and liver tissue from Control (CONT) and Adriamycin(ADR) groups animals. Data are expressed as mean ±SEM of 6 animals in CONT group and nine in ADR group.*) Represents significantly different ($p<0.05$) from the control

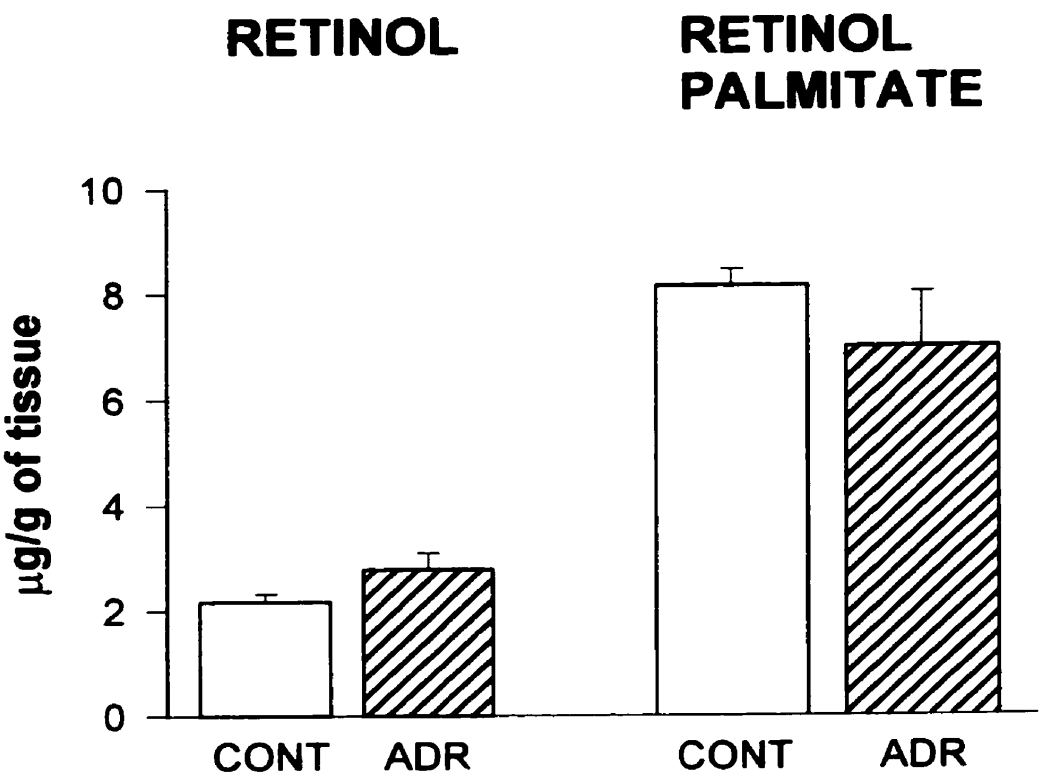


Figure 9: Retinol and retinol palmitate levels in the kidneys of Adriamycin-treated (ADR) and Control (CONT) groups. Data are expressed as mean ± SEM of 6 animals in CONT group and 9 in ADR group.

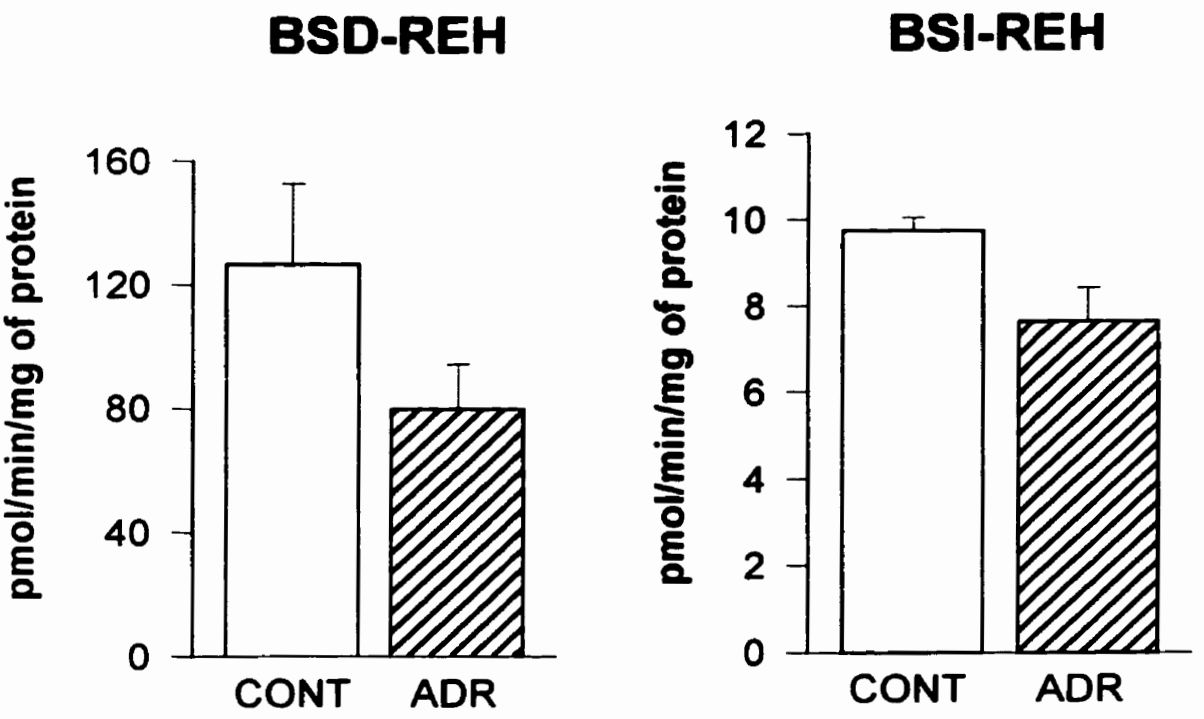


Figure 10: Bile salt dependent (BSD) and independent (BSI) retinol ester hydrolase (REH) enzyme activities in the liver of Control (CONT) and Adriamycin (ADR) groups. Data are expressed as mean ± SEM of 6 animals in CONT group and 9 in ADR group.

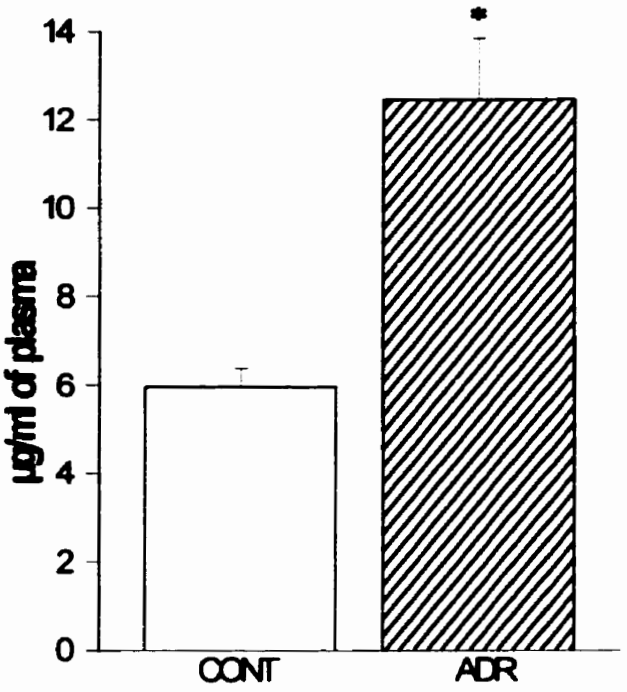


Figure 11: Alpha-tocopherol levels in plasma from control (CONT) and Adriamycin-treated (ADR) groups. Data are expressed as mean ± SEM of 6 animals in CONT group and 9 in ADR group.*) Represents significantly difference ($p<0.05$) from the control group.

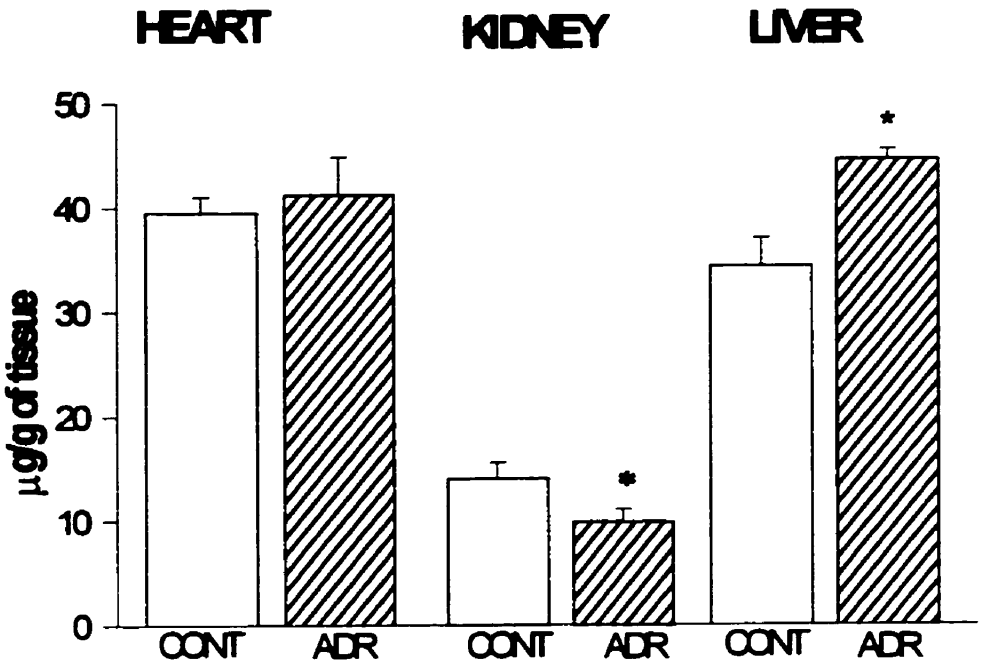


Figure 12: Alpha-tocopherol levels in heart, kidneys and liver of Control (CONT) and Adriamycin-treated (ADR) groups. Data are expressed as mean ± SEM of 6 animals in CONT group and 9 in ADR group.*)represents significantly difference ($p<0.05$) form the control group.

VI. DISCUSSION

Adriamycin, a potent antineoplastic drug, has been widely used for 3 decades for the treatment of a large variety of soft and solid human malignancies. The early enthusiasm about its beneficial effects was shadowed by the development of acute (Lefrak et al., 1973; Arena E. et al., 1972) and chronic (Buja et al., 1973; Lefrak et al., 1973) side effects. The acute side effects, consisting of myelosupression, nausea, vomiting and arrhythmias, are reversible and/or clinically manageable (Singal and Iliskovic, 1998).

However, the major problem due to adriamycin administration is the development of chronic side effects that can lead to development of dilated cardiomyopathy which can ultimately lead to congestive heart failure (Buja et al., 1973; Lefrak et al., 1973). Some of the cardiomyopathic changes related to the use of this drug have been reported to occur many years after the treatment regimen that included adriamycin (Steinherz et al., 1995). The risk of developing heart failure remains a life long threat in adriamycin-treated patients, especially in those where cumulative dose exceeds 550 mg per square meter of body surface (Singal and Iliskovic, 1998). Due to its toxicity, doses of adriamycin are carefully controlled and patients are advised to undergo a screening follow-up for the early signs of this drug-induced cardiomyopathy (Singal and Iliskovic, 1998).

Although the exact mechanism of adriamycin toxicity is not known, several subcellular changes are thought to play the role in this process (Singal et al., 1987). Most of the laboratory data available appears to support the view that there occurs an increase in oxidative stress and decrease in antioxidant reserve in the hearts from adriamycin treated animals (Singal et al., 1998). These changes appear to play an important role in the

pathogenesis of this cardiomyopathy (Doroshow et al., 1983). The present study focuses on the characterization of the nonenzymatic antioxidant changes in the retinol, retinyl esters and vitamin E in the heart. Since retinol levels are closely regulated by the storage organs (Palace et al., 1999), in our study we also focused on the metabolism of retinol in plasma, liver and kidney.

Congestive heart failure was induced in rats using a previously established model of adriamycin administration (Siveski-Iliskovic et al., 1995). After three weeks of post treatment, all animals exhibited a depression in the cardiac function. Depressed cardiac function was proven by the decrease in aortic systolic and peak ventricular pressures and an increase in end diastolic ventricular pressure. The latter change is viewed as the hallmark of congestive heart failure. This diagnosis was also confirmed by the presence of dyspnea and ascites in the ADR group. The development of this drug-induced heart failure could be caused by the increased oxidative stress, which in combination with a decreased antioxidant reserve could lead to damage of the heart muscle (Singal et al., 1987). This damage can become so extensive which might interfere with the normal heart function leading to pathological condition.

Retinol represents one of the most potent naturally occurring non-enzymatic antioxidants (Burton and Ingold 1984). In our study, the development of adriamycin induced heart failure was followed by the decrease in the retinol levels in the heart. However this decrease did not reach the level of statistical significance when compared with control. The decrease in the heart retinol levels were also followed by the increase in the heart levels of radio labeled retinol in the ADR group(81%). This indicates increased utilization of retinol which is counteracted by the increased uptake from the plasma pool (Palace et al., 1999a).

The unchanged retinol levels in the plasma of ADR group animals and increased levels of radio labeled retinol could be explained by some strict maintenance of this vitamin within a narrow range by its mobilization from the storage organs (liver and kidneys). In a previous study, Palace et al. (1999a) showed that retinol levels were decreased in the hearts of animals that survived myocardial infarction. This change was followed by the increased uptake of radio labeled retinol by the heart (Palace et al., 1999a). Plasma serves as a physiological pool from which vitamin A can be used for storage, or it can be mobilized to the organs where it is utilized.

In order to evaluate the metabolism of vitamin A, we also followed the levels of retinol in its storage organs. Liver and kidneys are the main storage organs of retinol, where it is mainly stored as retinol esters (retinol palmitate). The development of adriamycin-induced cardiomyopathy was characterized by a significant decrease in retinol levels in the liver. This was followed by the significant decrease in the levels of retinol palmitate (39%) and increased levels of radio labeled retinol (103%). These data suggest that retinol was probably mobilized from the liver in order to maintain the levels in the plasma pool (Palace et al., 1999a). In order to assess the mobilization of the retinol from the liver, we also examined the activity of retinol mobilizing enzymes: bile salt dependent and bile salt independent retinol ester hydrolases. These enzymes mobilize retinol by hydrolyzing it from retinyl esters (Harrison, 1993). Since activities of these enzymes in the ADR group were decreased, it is possible that there is a biphasic response indicating an increase in early stages followed by a decrease in late stages of failure. In this regard, a compensatory increase in these hydrolytic enzymes in the liver has been reported at mild to moderate stages of heart failure (Palace et

al., 1997). The decrease in late stages can be explained by the hepatotoxicity of adriamycin (Llesuy et al., 1990) and the development of congestive heart failure that is characterized by the congestive liver and the presence of ascites in the abdomen.

The kidney represents another important retinol storage organ. The kidney contribute in the maintenance of plasma levels of retinol in a narrow range by recapturing retinol that is not bound to its transport complex (transthyretin and retinol binding protein) and storing it as retinyl esters in the stellate cells. The development of adriamycin-induced cardiomyopathy was followed by the unchanged levels of retinol in kidneys. Although retinol and retinol ester concentration in the kidney in the ADR group was not changed, radio labeled retinol was increased by about 78%. These data also indicate increased mobilization of retinol during the development of adriamycin-induced congestive heart failure.

In this study, α-tocopherol in the heart in the ADR group was unchanged. Interestingly, in the plasma, as well as in the liver there was a significant increase in the α-tocopherol levels. In contrast, a significant decrease in its levels in the kidneys was noted. Under the conditions of increased oxidative stress, this pattern of change in the body redistribution of α-tocopherol may suggest some defect in the utilization of α-tocopherol in the heart. This contention is supported by the observations that vitamin E supplementation was ineffective in modulating adriamycin cardiomyopathic changes under chronic conditions (Singal et al., 1995; Van Vleet et al., 1980; Breed et al., 1980).

In conclusion, in spite of the maintenance of retinol levels in the heart, its increased utilization in the organ was indicated by an increased uptake of radio labeled retinol. This study also suggests that retinol levels in the heart and plasma may be strictly maintained, even

in severe pathological states such as adriamycin cardiomyopathy. The maintenance of retinol in a small concentration range may be achieved by its increased mobilization from the storage organs such as the liver. This study shows that under a condition of increased oxidative stress during adriamycin-induced congestive heart failure, there is an increased consumption of vitamin A. Further studies are required to determine the precise mechanism of its increased utilization in the heart as well as its mobilization from the storage organs.

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