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Full Length Research Paper

Cardiotoxicity induced by cyclophosphamide in rats: Protective effect of curcumin

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The possible protective effects of curcumin have been addressed in the current study. Cardiotoxicity was induced by challenging male Swiss albino rats with a single dose of cyclophosphamide (150 mg/kg, i.p.). Curcumin (200 mg/kg, i.p.) was administered for 8 consecutive days followed by a single dose of cyclophosphamide. Cardiotoxicity was well characterized morphologically and biochemically. The hallmark of this toxicity was marked congestion and edema in rat cardiac tissues, as well as severe inflammation in the cardiac muscles. Lymphocytic infiltration was also observed and determined by histopathological examination. Prior administration of curcumin ahead of cyclophosphamide challenge improved all the biochemical and histological alterations induced by the cytotoxic drug. Based on these broad findings, it could be concluded that curcumin has proven protective efficacy in this cyclophosphamide myocarditits model, possibly through modulating the release of inflammatory endocoids, namely tumor necrosis factor-alpha and nitric oxide, improving the energy status and restoring the oxidant/antioxidant balance.

Keywords: Curcumin, cyclophosphamide, cardiotoxicity, oxidative stress.

INTRODUCTION

Cyclophosphamide (CYP) is an alkylating agent with potent antineoplastic and immunosuppressive properties and possibly the most widely used anticancer drug (Gershwin et al., 1974; Tew et al., 1996). Cardiotoxicity associated with high-dose CYP has been described as a complication of several therapeutic regimens (Gottdiener et al., 1981; Birchall et al., 2000; Brockstein et al., 2000; Kamezaki et al., 2002). The incidence of fatal cardiomyopathy varies from 2.0% to 17.0%, depending on the different regimens and patient populations (Taniguchi, 2005).

In contrast to cardiomyopathy occurring months to years after high cumulative doses of anthracyclines, CYP-induced cardiomyopathy occurs within the initial 2 or 3 wk after treatment (Nieto et al., 2000; Taniguchi, 2005).

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Appelbaum et al. (1976) observed acute heart failure 5 to 9 d after treatment in 4 of 15 patients treated with CYP 45 mg · kg_1 · d_1 for 4 d. Postmortem examination of the heart revealed fibrin microthrombi in capillaries and fibrin strands within myocytes. Goldberg et al., 1986 reported congestive heart failure in 17% of patients treated with CYP at 50 mg · kg_1 · d_1 for 4 d, with a 43% mortality rate. These investigators observed a significant increase in the incidence of CYP-induced cardiac toxicity in patients receiving a dose of 1.55 g · m_2 · d_1 for 4 d. Acute heart failure secondary to cardiotoxicity has been reported about 1 wk after CYP administration, and the incidence rate is about 20% and mortality about 8% after bone marrow transplantation (Gottdiener et al., 1981; Goldberg et al., 1986).

Gottdiener et al. (1981) reported congestive heart failure in 28% of patients treated with 180 mg/kg of CYP within 3 wk of CYP administration. The pathogenesis of CYP-induced cardiotoxicity is thought to involve direct endothelial damage, leading in turn to leakage of plasma proteins and erythrocytes (Taniguchi, 2005). The histologic findings indicate acute pericarditis and hemorrhagic myocarditis with fibrin platelet microthrombi

in capillaries and fibrin strands in the interstitium on ultrastructural examination (Birchall et al., 2000). Wall thickening due to interstitial edema and hemorrhage may decrease left ventricular diastolic compliance as left ventricular diastolic dysfunction and present as restrictive cardiomyopathy.

CYP is an inactive prodrug that requires metabolic activation by the cytochrome P-450 system. The process of CYP activation produces hydroxylated active metabolites, e.g., acrolein, phosphoramide mustard, and nitrogen mustard, believed to be toxic, or the inactive compound carboxyphosphamide (Tew et al., 1996). CYP metabolites can react with carboxyl (-C[O]OH), mercapto (-SH), amino (-NH2), phosphate (-PO3H2), and hydroxyl (-OH) groups and can form cross-links with DNA and proteins (Slavin et al.,1975 ; Fleming, 1997; Nieto et al., 2000). CYP is believed to exert its cardiotoxic effects through damage of the endocardial capillary endothelium, resulting in increased permeability and microthromboses and extravasation of plasma and red blood cells into the myocardium (Fleming, 1997; Slavin et al., 1975). CYP administration has been associated with increased lipid peroxidation and significant depletion of antioxidant molecules, including glutathione (GSH), catalase, and superoxide dismutase (Patel and Block, 1985; Patel, 1987; Dorr and Lagel, 1994).

Multiple clinical studies have suggested that the use of antioxidants in combination with chemotherapy and irradiation prolong the survival time of patients compared with expected outcome without antioxidant supplements (Singh and Lippman, 1988; Manda and Bhatia, 2003; Sudharsan et al., 2006).

Curcumin (diferuloylmethane) [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is the principal component of the turmeric pigment of *Curcuma longa* which is commonly used as a spice and food-colouring agent (Ammon and Wahl, 1991). In the Indian subcontinent and Southeast Asia, turmeric has traditionally been used as a treatment for inflammation, skin wounds and tumours. Clinical benefits of curcumin have yet to be confirmed. However, in preclinical animal models, curcumin has shown antioxidant (Ak and Gülçin, 2008), antidiabetic (Meghana et al., 2008), antineoplastic (Yoysungnoen et al., 2008) and antiinflammatory (Jacob et al., 2007) properties. Due to such pleiotropic beneficial effects of curcumin, the current work was conducted in an attempt to address whether or not the turmeric pigment would exert protective effects in this cyclophosphamide induced cardiotoxicity.

Aim of the work

The aim of the work is to study the possible cyto-protective effect of curcumin against cyclophosphamide induced cardiotoxicity.

MATERIALS AND METHODS

Chemicals and Drugs

Cyclophosphamide was supplied as vials from Baxter Oncology (Düsseldorf, Germany). Curcumin was purchased as orange-yellow powder from Fluka Chemical Co. (Steinheim, Germany). All other chemicals were of the highest grade commercially available.

Animals

Male Swiss albino rats weighing 150-200 g were used in all experiments. Animals were maintained under standard conditions of temperature and humidity with regular light/dark cycle and allowed free access to food (Purina Chow) and water. All animal experiments were conducted according to the regulations of the Committee on Bioethics for Animal Experiments of Riyadh colleges of dentistry and pharmacy.

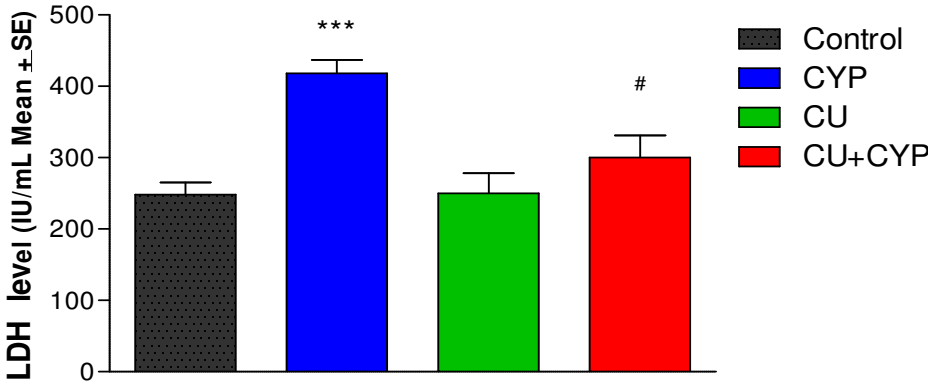
Design of the work

A total of 24 male Swiss albino rats were allotted to four groups with six animals in each. Treatment regimens were as such: The first group received normal saline (2 ml/kg, i.p.) for 8 consecutive days and served as control. A second group was administered curcumin (200 mg/kg, i.p.) for 8 consecutive days. A third group was given saline as before and then challenged with a single dose of cyclophosphamide (150 mg/kg, i.p.). A fourth group received curcumin as before and followed thereafter with cyclophosphamide challenge (150 mg/kg, i.p.). Forty-eight hours after cyclophosphamide treatment, animals were euthanized by cervical dislocation and retroorbital blood samples were withdrawn under light ether anaesthesia. Serum was separated and hearts were dissected out, plotted dry on filter paper and kept in 10% formol saline prior to histopathological examination.

Biochemical assessment of cardiotoxicity

Assessment of serum lactate dehydrogenase LDH and creatine phosphokinase CPK

Serum was separated by centrifugation at 4000 rpm for 4 min and stored at –20˚C until analysis. LDH and CPK levels were assayed using commercially available reagents (bioMerieux, France) based on the method of Buhl and Jackson, 1978 and Gruber, 1979 respectively.



CU (200mg/kg/day i.p.) was given for 8days before CYP (150mg/kg i.p.). Significantly different from control group # Significantly different from CYP #* P<0.05 ##** P<0.01 ###*** P<0.001

Figure 1. Effects of CU on elevated serum enzymes LDH activities induced by CYP.

Determination of reduced glutathione and lipid peroxidation in cardiac tissues

The tissue levels of the acid soluble thiols, mainly GSH, were assayed spectrophotometrically at 412 nm, according to the method of Ellman, 1959 using a Shimadzu (Tokyo, Japan) spectrophotometer. The contents of GSH were expressed as mol g−1 wet tissue. The degree of lipid peroxidation in cardiac tissues was determined by measuring thiobarbituric acid reactive substances (TBARS) in the supernatant tissue from homogenate Ohkawa et al., 1979. The homogenates were centrifuged at 3500 rpm and supernatant was collected and used for the estimation of TBARS. The absorbance was measured spectrophotometrically at 532 nm and the concentrations were expressed as nmol TBARS g−1 wet tissue.

Determination of total nitrate/nitrite (NO(x)) concentrations in cardiac tissues

Total nitrate/nitrite (NO(x)) was measured as stable end product, nitrite, according to the method of Miranda et al. 2001. The assay is based on the reduction of nitrate by vanidium trichloride combined with detection by the acidic griess reaction. The diazotization of sulfanilic acid with nitrite at acidic pH and subsequent coupling with N-(10 naphthyl)-ethylenediamine produced an intensely colored product that is measured spectrophotometrically at 540 nm. The levels of NOx were expressed as mol g−1 wet tissue.

Histopathologic studies

Specimens for light microscopy were immediately fixed in 10% buffered formalin and subsequently embedded in paraffin media. Several 6-_m tissue sections were cut from each paraffin block and mounted on glass slides. The slides were stained with hematoxylin and eosin. Histologic evaluation of the cardiac specimens was made in a blinded fashion. Pathology was graded based on the presence and severity of the following parameters, including edema, leukocytic infiltration, muscle necrosis, chronic inflammation, and fibrosis edema, leukocytic infiltration, muscle necrosis, chronic inflammation, and fibrosis. Grading for each component was performed by using a semi-quantitative scale where 0 was normal and 1–4_ represented mild through severe abnormalities. The total cardiac injury score for each heart was a calculated as an average of all the component injury scores.

Statistical Analysis

Data are expressed as (means ± SEM). Statistical comparison between different groups were done using one way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test to judge the difference between various groups. Significance was accepted at P< 0.05.

RESULTS

Cardiovascular Effects

Serum lactate dehydrogenase LDH and creatine phosphokinase CPK were significantly elevated (P<0.001) after injection of CYP reaching 418+18and 622+29U/L respectively as compared with the control group. Combined CYP treatment with curcumin decreased significantly enzymes activities (P<0.001) (Figure 1 and 2).

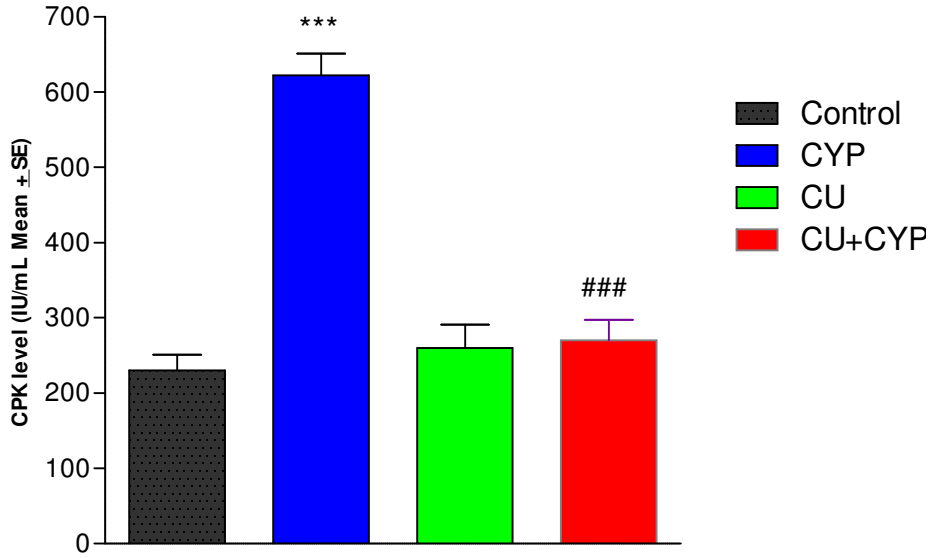


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Figure 2. Effects of CU on elevated serum enzymes CPK activities induced by CYP.

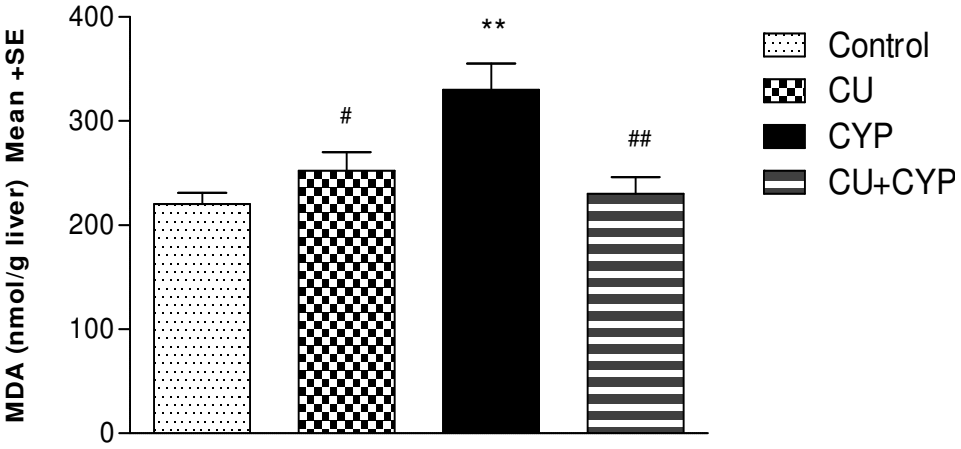

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Figure 3. Effects of CYP, CU and their combination on the levels of thiobarbituric acid reactive substance (MDA) in rat cardiac tissues.

Oxidative stress biomarkers

Figure 3, 4 and 5 show the effects of CYP, curcumin and their combination on oxidative stress biomarkers namely thiobarbituric acid reactive substance (MDA) , GSH , and NOx in cardiac tissues respectively. CYP resulted in a significant 30% decrease in GSH and a significant 42% and 99% increase in TBARS and NO(x), respectively, as compared to the control group. Combined CYP treatment with curcumin decreased significantly MDA, NO(x), (P<0.001) and restore GSH level in cardiac tissues to the control values.

Cardiac pathology

Cardiac pathologic injury scores were determined for the studied groups (Table 1). The pathologic injury score was elevated only the CYP group. The cardiac injury scores correlated with the presence of muscle necrosis, edema, and inflammation seen in CYP group (Figure 6 and 7) compared with the normal cardiac muscle tissue

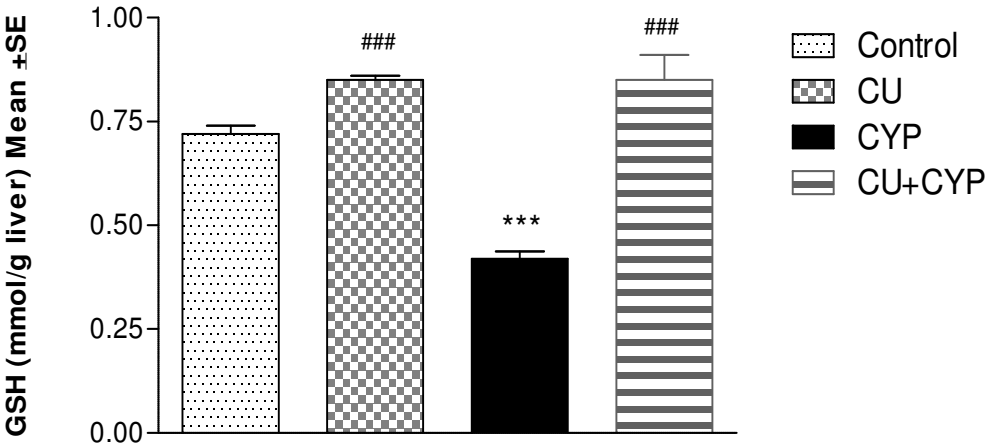

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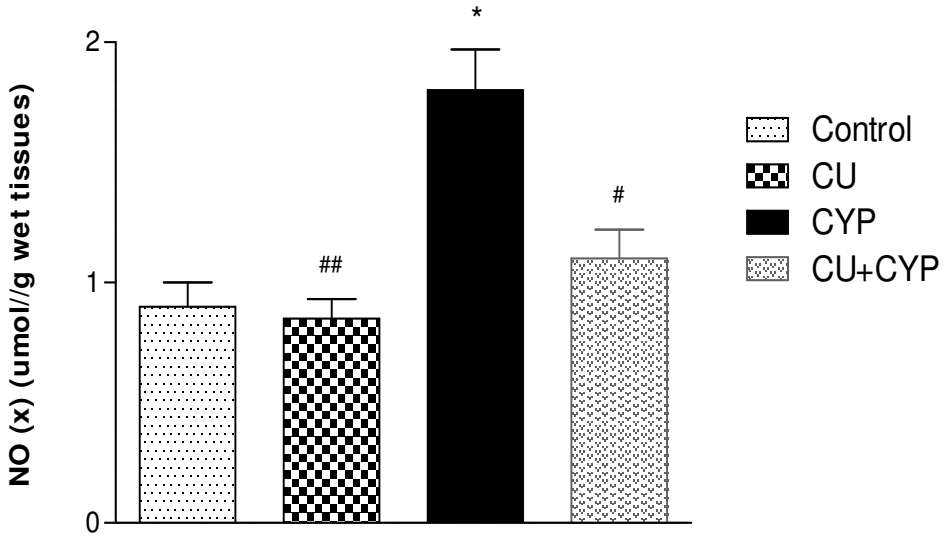
Figure 4. Effects of CYP, CU and their combination on the levels of reduced glutathione in rat cardiac tissues.



CU (200mg/kg/day i.p.) was given for 8days before CYP (150mg/kg i.p.).
Significantly different from control group # Significantly different from CYP
#* P<0.05 ##** P<0.01 ###*** P<0.001

Figure 5. Effects of CYP, CU and their combination on the levels of total nitrate/nitrite in rat cardiac tissues.

Table 1. Schematic representation of cardiac injury score determined by scoring of 10 random fields on slides of hearts stained with hematoxylin and eosin at 48 h.

Group	Histopathological score
Control group	0.4 ± 0.2
Curcumin group	2 ± 0.3
cyclophosphamide group	$^{*}8 \pm 0.7$
curcumin and clophosphamide group	$^{\#}4 \pm 0.6$

The injury was score for five parameters: edema, leukocytic infiltration, muscle necrosis, inflammation, and fibrosis. * Significant elevation above the control group, # significant elevation above the control groups.

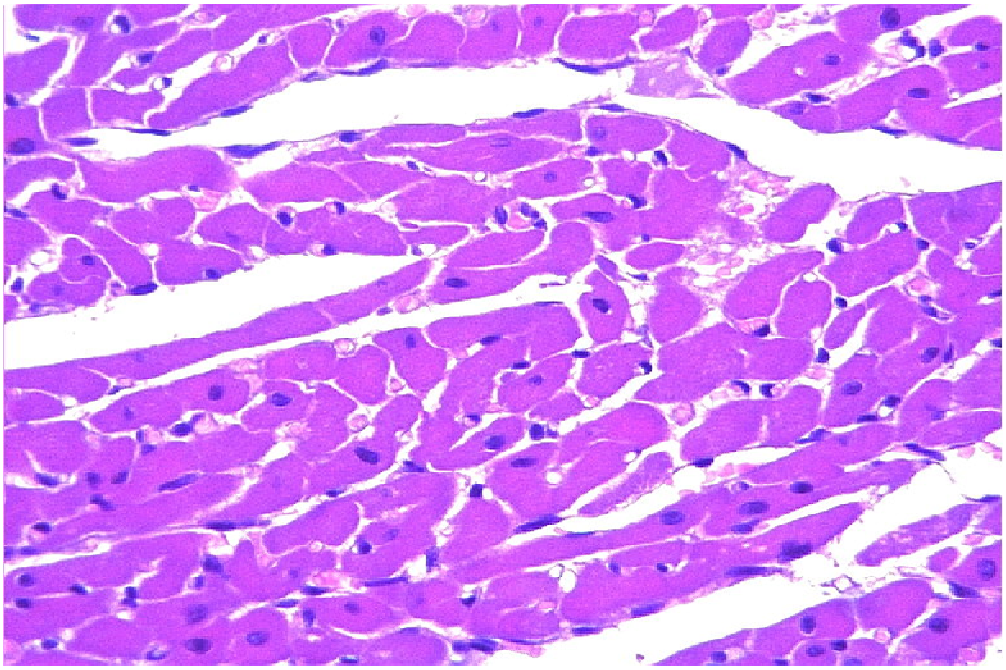


Figure 6. (Control Group)-Heart muscle normal X200.

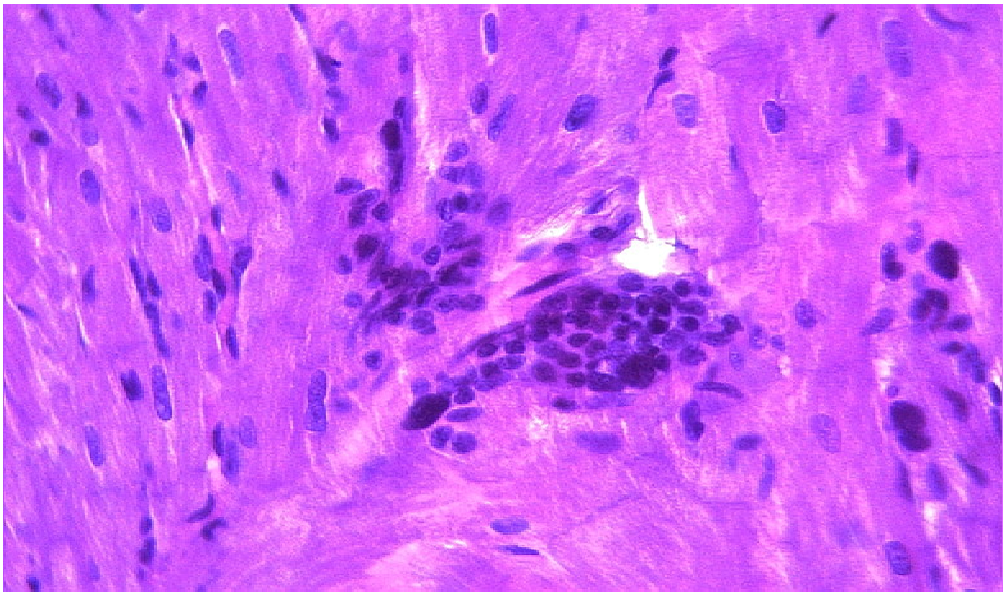


Figure 7. (CYP Group)--Heart muscle inflammatory cells more severe X400.
Sections of heart ventricle from the group that received 150 mg/kg of cyclophosphamide reveal injured myocytes with scattered coagulative changes and thin bands of contraction necrosis. Magnification 400_

observed in the control group (Figure 8 and 9).

DISCUSSION

Cyclophosphamide is a cytotoxic drug that is highly effective in the treatment of various human cancers particularly lymphomas and some types of leukaemia and autoimmune diseases. The clinical utility of the oncolytic agent has been hampered by dose-limiting toxicities; one of the most frequent complications is myocarditis (Hu et al., 2008). Due to the pleiotropic effects of curcumin, it

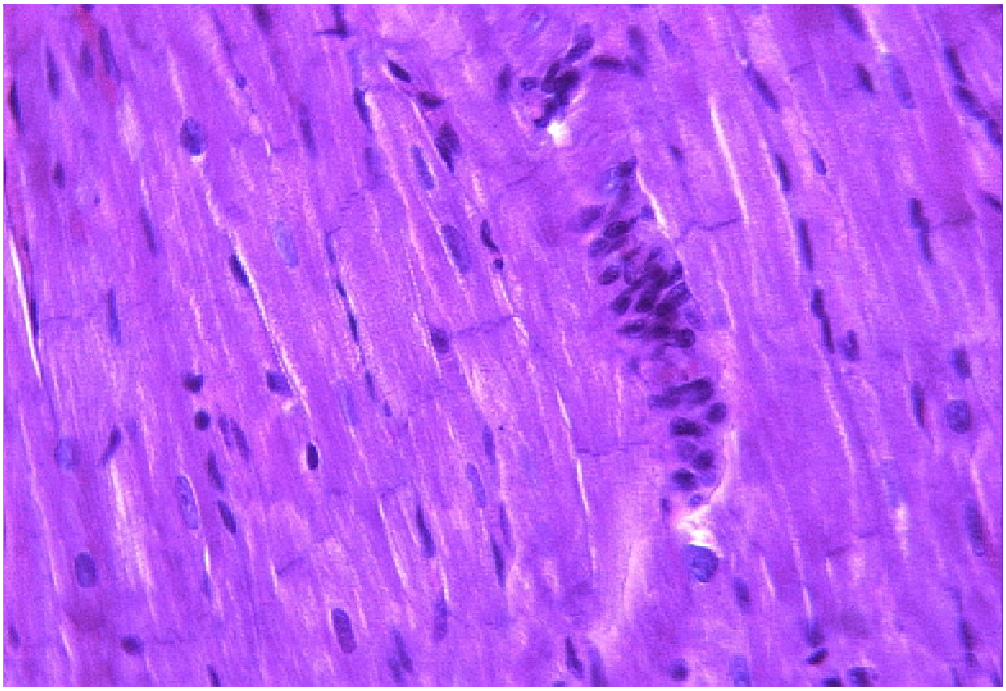


Figure 8. (CU-CYP Group)-Heart muscle inflammatory cells X400.
Sections of heart ventricle from the group that received curcumin plus 200 mg/kg of cyclophosphamide reveal individual cardiac muscle cells arranged in diffuse bundles in a connective tissue framework. Individual myocytes are seen in cross section to be well stained and preserved. They have centrally located nuclei with abundant cytoplasm outlined by distinct and intact cell walls. The myocytes essentially appear normal. Magnification 200.

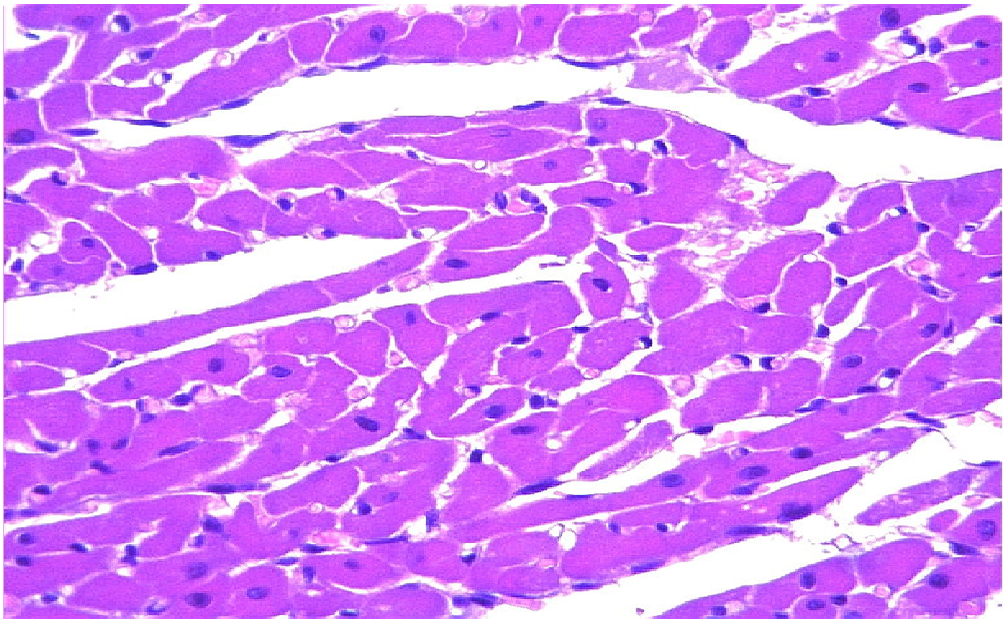


Figure 9. (Curcumin Group)-Heart muscle normal X400.

was a suitable candidate to be tested in the present work for any possible protective effects (Arafa, 2009).

Cyclophosphamide challenge induced cardiotoxicty that was well characterized morphologically and biochemically. Cardiotoxicty was manifested by marked congestion, oedema and extravasation in the cardiac

tissues, as well as a marked leucocytic infiltration as determined by macroscopic and histopathological examination (Lieber et al., 1984 ; Malley and Vizzard, 2002). Cyclophosphamide markedly increased the serum level of LDH and CPK enzyme activites. Similar results were previously documented (Wong et al., 2000; Linares-Fernández and Alfieri, 2007).

Though the exact pathogenesis whereby cyclophosphamide induces toxicity is mediated through its toxic metabolite, acrolein, the molecular events underlying such toxicity are yet to come. The pathogenetic pathways may include oxidative damage, release of some inflammatory endocoids such as cytokines and nitric oxide as well as poly (adenosine diphosphate-ribose) polymerase activation (Dang et al., 2008).

Curcumin has proven protective efficacy when administered prior to cyclophosphamide challenge as shown morphologically and biochemically. The turmeric pigment prevented the severe inflammation and congestion. Only slight extravasation and leucocytic infiltration were observed (Arafa, 2009).

Similar findings were reported for curcumin in other inflammatory conditions including acute pancreatitis (Xu et al., 2001) and acute liver damage (Manesh and Kuttan, 2005). Curcumin regulated both the hypokalaemia and hyponatraemia induced by cyclophosphamide. Likewise, Babu and Srinivasan (Sheeja and Kuttan, 2006) have demonstrated that feeding curcumin to diabetic rats prevented the urinary loss of potassium and sodium and consequently corrected hypokalaemia and hyponatraemia.

Curcumin is a well known inducible nitric oxide synthase inhibitor (Takahashi et al., 2011). The role of nitric oxide in CYP induced cardiotoxicity has been recently considered. Nitric oxide has been reported to be involved in diverse physiological and pathophysiological processes including host immune defense, vasoregulation and the pathogenesis of diabetes (Guzel et al., 2012). Nitric oxide synthase (NOS), an enzyme that is involved in the synthesis of NO has been shown to be activated in the inflammatory lesion (Mouzaoui et al., 2012). There are at least three types of NOS, the constitutive cNOS , the endothelial eNOS and the inducible iNOS []. Recently, the iNOS was expressed in the myocardium and large amount of NO and superoxide was produced in the rat hearts with experimental myocarditits (Manikandan et al., 2011). The production of excess amount of iNOS and in turn NO reacts with superoxide and form a peroxynitrite, converting tyrosine in the myocardium to nitrotyrosine and leading to myocardial injury in the autoimmune myocarditis in rats . Peroxinitrite formed from NO is a powerful oxidant and cause tissue damage (Mito et al., 2011).

Based on these broad observations, it could be concluded that curcumin has proven protective efficacy in this cyclophosphamide-induced cardiotoxicity. Such protection is possibly mediated through modulation of the release of some inflammatory endocoids, namely TNF-α and nitric oxide, improving the energy status and regulating the oxidant/antioxidant balance.

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