

Probucol attenuates cyclophosphamide-induced oxidative apoptosis, p53 and Bax signal expression in rat cardiac tissues

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Key words: cyclophosphamide, probucol, apoptosis, ATP, oxidative stress

Cyclophosphamide (CP) is a widely used drug in cancer chemotherapy and immunosuppression, which could cause toxicity of the normal cells due to its toxic metabolites. Probucol, a cholesterol-lowering drug, acts as potential inhibitor of DNA damage and shows to protect against doxorubicin-induced cardiomyopathy by enhancing the endogenous antioxidant system including glutathione peroxidase, catalase and superoxide dismutase. This study examined the possible protective effects of probucol, a lipid-lowering compound with strong antioxidant properties, against CP-induced cardiotoxicity. This objective could be achieved through studying the gene expression-based on the possible protective effects of probucol against CP-induced cardiac failure in rats. Adult male Wistar albino rats were assigned into four treatment groups: Animals in the first (control) and second (probucol) groups were injected intraperitoneally with corn oil and probucol (61 mg/kg/day), respectively, for two weeks. Animals in the third (CP) and fourth (probucol plus CP) groups were injected with the same doses of corn oil and probucol (61 mg/kg/day), respectively, for one week before and one week after a single dose of CP (200 mg/kg, I.P.). The p53, Bax, Bcl2 and oxidative genes signal expression were measured by real time PCR. CP-induced cardiotoxicity was clearly observed by a significant increase in serum creatine phosphokinase isoenzyme (CK-MB) (117%), lactate dehydrogenase (LDH) (64%), free (69%) and esterified cholesterol (42%) and triglyceride (69%) compared to control group. In cardiac tissues, CP significantly increases the mRNA expression levels of apoptotic genes, p53 with two-fold and Bax with 1.6-fold, and decreases the anti-apoptotic gene Bcl2 with 0.5-fold. Moreover, CP caused downregulation of antioxidant genes, glutathione peroxidase, catalase, and superoxide dismutase and increased the lipid peroxidation and decreased adenosine triphosphate (ATP) (40%) and ATP/ADP (44%) in cardiac tissues. Probucol pretreatment not only counteracted significantly the CP-induced increase in cardiac enzymes and apoptosis but also induced a significant increase in mRNA expression of antioxidant enzymes and improved ATP, ATP/ADP, glutathione (GSH) in cardiac tissues. In conclusion, data from the present study suggest that probucol prevents the development of CP-induced cardiotoxicity by a mechanism related, at least in part, to its ability to increase mRNA expression of antioxidant genes and to decrease apoptosis in cardiac tissues with the consequent improvement in mitochondrial oxidative phosphorylation and energy production.

Introduction

The clinical use of cyclophosphamide (CP), a commonly used oxazaphosphorine alkylating agent, has been extended from neoplastic diseases to organ transplantation and diverse disorders and as an immunosuppressive agent.¹⁻³ The major limitation of CP is the injury of normal tissue, leading to multiple organ toxicity.^{1,2} The important factor for both therapeutic and toxic effects of CP is the requirement of metabolic activation by hepatic microsomal cytochrome P450 mixed functional oxidase system.^{3,4} It is well documented that high therapeutic doses of CP could cause a lethal cardiotoxicity that has a combination of symptoms and signs of myopericarditis leading to fatal

complications such as congestive heart failure, arrhythmias, cardiac tamponade and myocardial depression.⁵ The pathogenesis of CP-induced acute cardiotoxicity was attributed to the increase in free oxygen radicals and the decrease in the antioxidant defense mechanism by CP in the heart.⁶ Hypercholesterolemia, hypertriglyceridemia and impaired secretion of heart lipoprotein lipase have been reported in CP-treated rabbits.^{7,8} Lipid homeostasis plays a central role in the pathogenesis of primary and/or secondary alterations of lipid metabolism pathways in various conditions lead to myocardial lipid accumulation and lipotoxic cardiomyopathy.⁹

Oxidative stress has been widely shown to regulate apoptosis and exerts both agonistic and antagonistic effects on apoptotic

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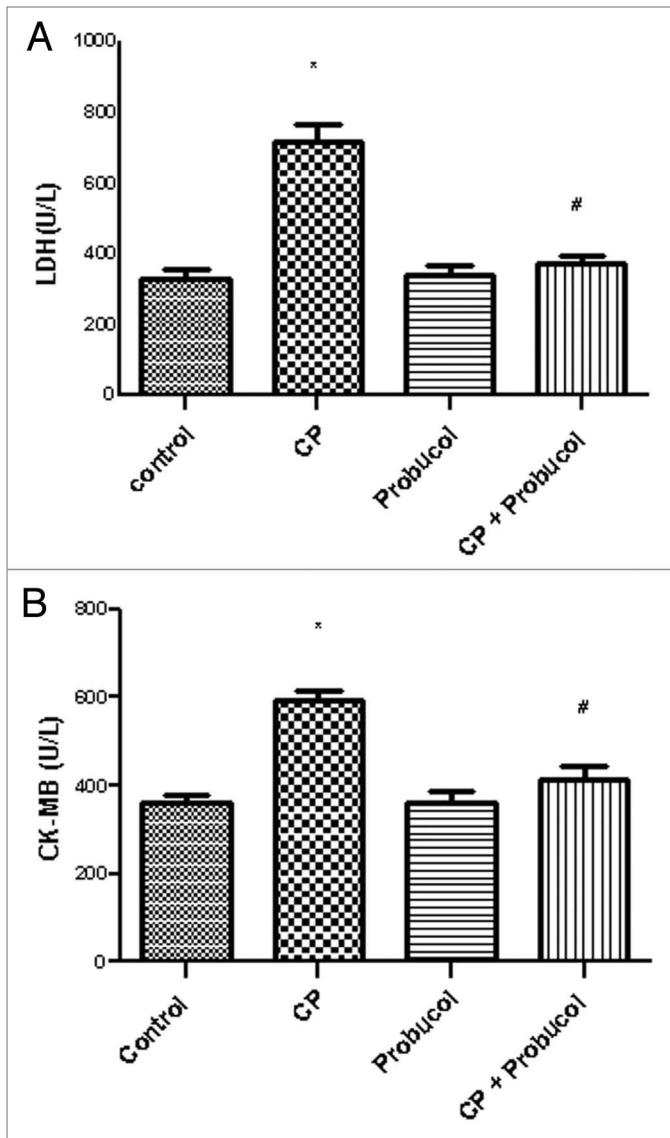


Figure 1. Effect of cyclophosphamide (CP), probucol and cyclophosphamide (CP) plus probucol on serum lactate dehydrogenase (LDH) (A) and creatine phosphokinase isoenzyme (CK-MB). Rats were randomly divided into four different groups of ten animals each: control, CP, probucol and probucol plus CP. Control rats received corn oil for two weeks. Rats in CP group were injected with corn oil for one week before and one week after a single dose of CP (200 mg/kg, I.P.). Rats in group 3 (probucol group) were injected with probucol (61 mg/kg/day, I.P.) for two weeks. Rats in the fourth group (probucol plus CP group) received probucol (61 mg/kg/day, I.P.) for one week before and one week after a single dose of CP (200 mg/kg, I.P.). At the end of the treatment protocol, blood samples were obtained and serum was separated for measurement of LDH and CK-MB. Data are presented as mean \pm S.E.M. (n=10). * and # indicate significant change from control and CP, respectively, at $p < 0.05$ using ANOVA followed by Tukey–Kramer as a post ANOVA test.

signaling.¹⁰ It has been demonstrated to mediate cell proliferation and differentiation, which are considered the opposite of cell death by apoptosis. It is associated with p53-dependent cell cycle arrest, DNA repair and apoptosis.¹¹ Under oxidative stress a fraction of cellular p53 traffics to mitochondria and initiates

apoptosis. The release of mitochondrial Cyt-c can be triggered through p53-induced activation of Bax in a caspase-dependent manner.¹² Bcl2 is critical for regulation of apoptosis across diverse cell types and acts along intrinsic mitochondrial apoptosis pathway that is activated in response to a number of stress stimuli including oxidative stress.¹³

Probucol is a clinically used cholesterol-lowering drug.¹⁴ Beside its antioxidant properties, probucol was shown to protect against diabetes-associated¹⁵ and doxorubicin-induced cardiomyopathy^{16,17} by enhancing the endogenous antioxidant system including glutathione peroxidase, catalase and superoxide dismutase. In addition, probucol acts as potential inhibitor of DNA damage.^{18,19} Because DNA strands can be damaged directly by anticancer drugs or indirectly by the production of free radicals, it is reasonable to assume that changes in antioxidant enzyme activities following CP treatment could be the result of altered gene expression. To date, in the literature, there is no any study investigating the effect of probucol against CP-induced cardiotoxicity. Taken together, this prompted us to initiate this study to investigate the possible protective effects of probucol against CP-induced cardiac failure in rats, based on the expression levels of some genes.

Results

Intraperitoneal administration of a single dose of CP (200 mg/kg) induced severe biochemical changes as well as oxidative damage in cardiac tissue. Cyclophosphamide-induced cardiotoxicity was clearly observed in the current study by the increase in serum cardiotoxicity indices, CK-MB and LDH (Fig. 1). A single dose of CP resulted in a significant 117 and 64% increase in CK-MB (A) and LDH (B), respectively, compared to control group. Daily administration of probucol to CP-treated rats resulted in a complete reversal of CP-induced increase in CK-MB and LDH to the normal values.

Figure 2 shows the effects of CP, probucol, and their combination on the concentration of free cholesterol (A), esterified cholesterol (B) and triglycerides (C) in serum. Treatment with CP resulted in a significant 69, 42 and 69% increase in free cholesterol, esterified cholesterol and triglycerides respectively, compared to control group. Treatment with probucol for one week before and after a single dose of CP, completely reversed CP-induced increase in lipid profile to the normal values.

To investigate the effect of CP administration on mitochondrial function and energy production, the level of ATP and ATP/ADP ratio were measured in cardiac tissues (Fig. 3). A single dose of CP resulted in a significant 40 and 44% decrease in ATP (A) and ATP/ADP (B), respectively, as compared to control group. On the other hand, daily administration of probucol for two weeks resulted in a significant 56 and 239% increase in ATP and ATP/ADP, respectively, as compared to control group. Fascinatingly, administration of probucol to CP-treated rats resulted in a complete reversal of the CP-induced decrease in ATP and ATP/ADP in cardiac tissues to the normal values.

Figure 4 shows the effect of CP, probucol and their combination on the levels of oxidative biomarkers namely, GSH

Figure 2. Effect of cyclophosphamide (CP), probucol and cyclophosphamide (CP) plus probucol on serum-free cholesterol (A), esterified cholesterol (B) and triglycerides (C). Rats were randomly divided into four different groups of ten animals each: control, CP, probucol and probucol plus CP. Control rats received corn oil for two weeks. Rats in CP group were injected with corn oil for one week before and one week after a single dose of CP (200 mg/kg, I.P.). Rats in group 3 (probucol group) were injected with probucol (61 mg/kg/day, I.P.) for two weeks. Rats in the fourth group (probucol plus CP group) received probucol (61 mg/kg/day, I.P.) for one week before and one week after a single dose of CP (200 mg/kg, I.P.). At the end of the treatment protocol, blood samples were obtained and serum was separated for measurement of free cholesterol, esterified cholesterol and triglycerides. Data are presented as mean \pm S.E.M. (n=10). * and # indicate significant change from control and CP, respectively, at $p < 0.05$ using ANOVA followed by Tukey–Kramer as a post ANOVA test.

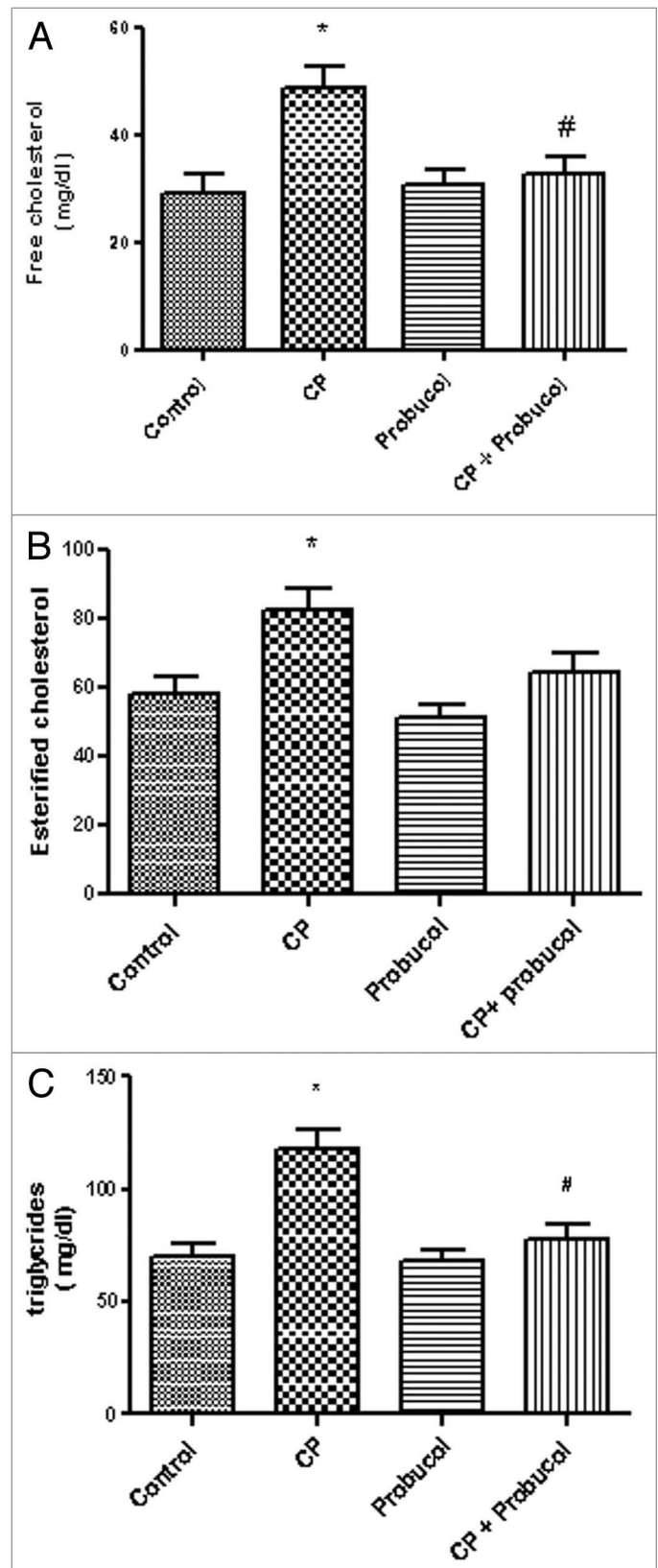
(Fig. 4A) and TBARS (Fig. 4B) in rat heart tissues. CP treatment resulted in a significant 51% decrease in GSH and 62% increase in TBARS as compared to control group. Daily treatment with probucol for two weeks resulted in a significant 71% increase in GSH in cardiac tissues as compared to control group. Interestingly administration of probucol in combination with CP resulted in a complete reversal of CP-induced increase in TBARS and decrease in GSH to the normal values.

Figure 5 shows the effect of CP, probucol, and their combination on the P53-apoptotic pathway, P53 (Fig. 5A), Bax (Fig. 5B) and Bcl2 (Fig. 5C), mRNA expression level in cardiac tissues. CP resulted in a significant two- and 1.6-folds increase in P53 and Bax mRNA expression level. In contrast, CP induced a significant 0.5-fold decrease in Bcl2 mRNA expression in cardiac tissues. Two weeks' treatment with probucol significantly decreased p53 and Bax mRNA expression and increased Bcl2 mRNA expression in cardiac tissues. Interestingly administration of probucol to CP-treated rats completely reversed the increase in P53 and Bax mRNA expression and the decrease in Bcl2 mRNA expression, induced by CP, to the control values.

Figure 6 shows the effects of CP, probucol, and their combination on the mRNA expression of antioxidant enzymes, glutathione peroxidase (Fig. 6A), catalase (Fig. 6B) and superoxide dismutase (Fig. 6C), in cardiac tissues. A single dose of CP resulted in a significant 0.6-, 0.6-, and 0.5-fold increases in mRNA expression of glutathione peroxidase, catalase, and superoxide dismutase, respectively, as compared to the control group. In contrast to CP group, a high expression of glutathione peroxidase, catalase, and superoxide dismutase mRNA expression was observed in probucol-treated rats as compared to control group. Probucol not only increased expression of antioxidant genes, but also completely reversed CP-induced decrease in the expression of these genes to the normal values.

Discussion

It is well documented that high therapeutic doses of CP can cause an acute form of cardiotoxicity within ten days of its administration.²⁰ Cellular mechanisms of CP-induced cardiotoxicity are thought to be mediated by an increase in free oxygen radicals through intracellular phosphoramidate mustard, the principal



alkylating metabolite of CP which affects endothelium and ion transport mechanisms.^{1,21,22} This study has been initiated to investigate the possible mechanisms whereby probucol could prevent the development of CP-induced cardiotoxicity.

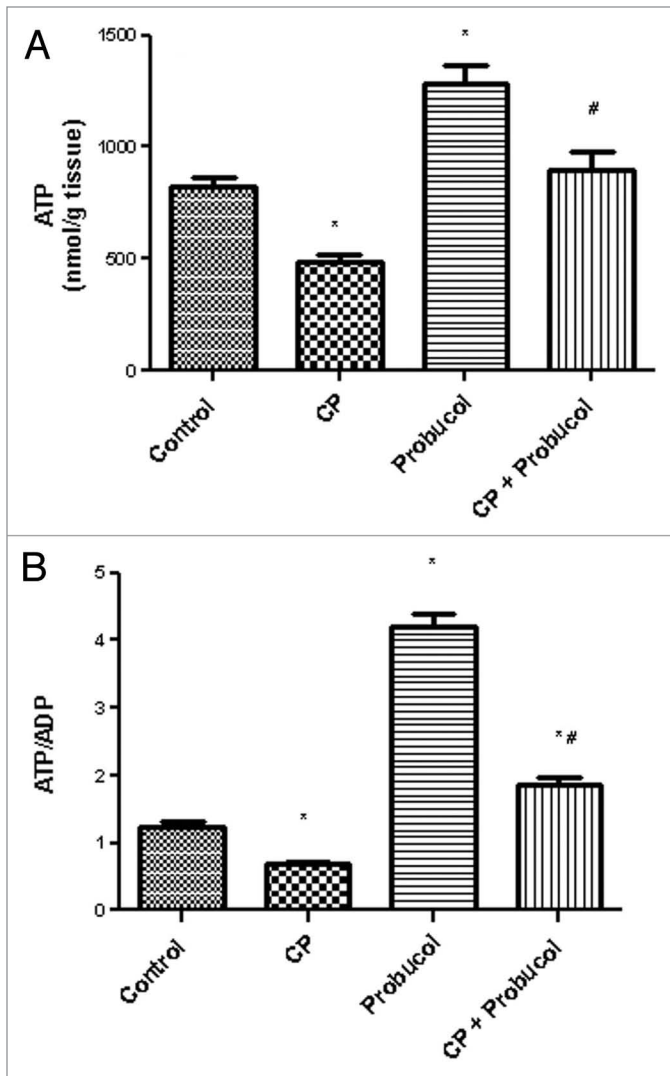


Figure 3. Effect of cyclophosphamide (CP), probucol and cyclophosphamide (CP) plus probucol on the levels of adenosine triphosphate (ATP) (A) and ATP/ADP (B) in cardiac tissues. Rats were randomly divided into four different groups of ten animals each: control, CP, probucol and probucol plus CP. Control rats received corn oil for two weeks. Rats in CP group were injected with corn oil for one week before and one week after a single dose of CP (200 mg/kg, I.P.). Rats in group 3 (probulcol group) were injected with probucol (61 mg/kg/day, I.P.) for two weeks. Rats in the fourth group (probulcol plus CP group) received probucol (61 mg/kg/day, I.P.) for one week before and one week after a single dose of CP (200 mg/kg, I.P.). At the end of the treatment protocol, animals were sacrificed, hearts were isolated and homogenized for measurement of ATP and ADP. Data are presented as mean \pm S.E.M. (n=10). * and # indicate significant change from control and CP, respectively, at $p < 0.05$ using ANOVA followed by Tukey–Kramer as a post ANOVA test.

In the current study, CP significantly increased serum cardiotoxicity enzymatic indices, LDH and CK-MB, and free cholesterol, esterified cholesterol and triglycerides in serum. Our results are in line with several authors²³⁻²⁷ who demonstrated a marked elevation of CK-MB, LDH, ALT, AST, cholesterol and triglycerides 10 days after administration of a single dose of CP (200 mg/kg). Increased activities of cardiac enzymes in serum are

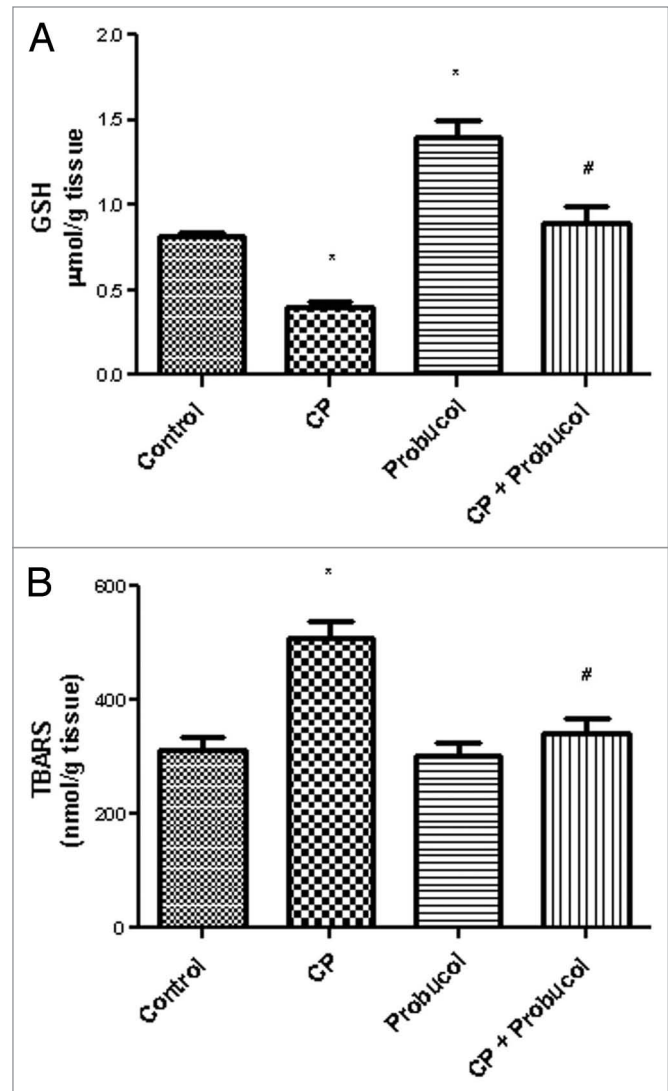


Figure 4. Effect of cyclophosphamide (CP), probucol and cyclophosphamide (CP) plus probucol on the levels of Glutathione (GSH) (A) and thiobarbituric acid reactive substances (TBARS) (B) in cardiac tissues. Rats were randomly divided into four different groups of ten animals each: control, CP, probucol and probucol plus CP. Control rats received corn oil for two weeks. Rats in CP group were injected with corn oil for one week before and one week after a single dose of CP (200 mg/kg, I.P.). Rats in group 3 (probulcol group) were injected with probucol (61 mg/kg/day, I.P.) for two weeks. Rats in the fourth group (probulcol plus CP group) received probucol (61 mg/kg/day, I.P.) for one week before and one week after a single dose of CP (200 mg/kg, I.P.). At the end of the treatment protocol, animals were sacrificed, hearts were isolated and homogenized for measurement of GSH and TBARS. Data are presented as mean \pm S.E.M. (n=10). * and # indicate significant change from control and CP, respectively, at $p < 0.05$ using ANOVA followed by Tukey–Kramer as a post ANOVA test.

well-known diagnostic indicators of cardiac injury.²⁸ Also, hypercholesterolemia, hypertriglyceridemia induced by CP, which are well-known risk factors in cardiovascular diseases, has been reported previously.²⁹ Increased generation of ROS by CP,²¹ may cause cellular cholesterol accumulation by increasing cholesterol biosynthesis and its esterification by decreasing cholesteryl ester

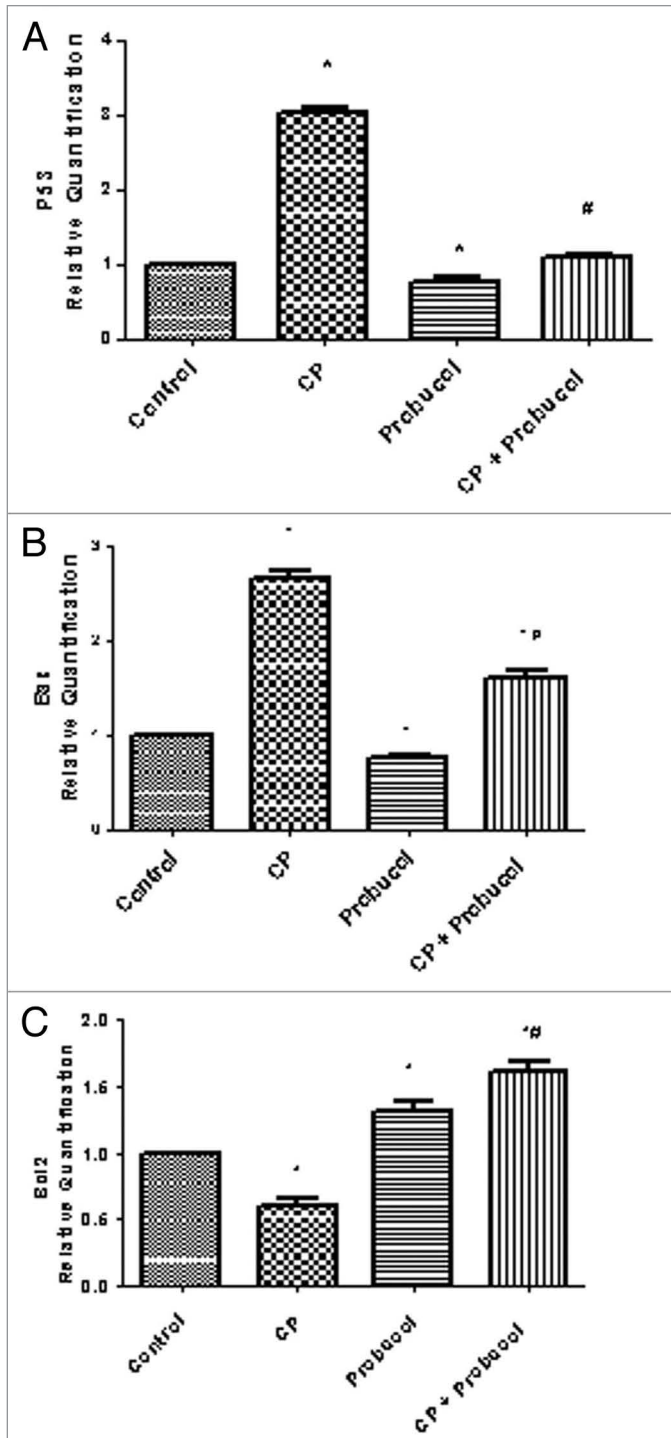


Figure 5. Effect of cyclophosphamide (CP), probucol and cyclophosphamide (CP) plus probucol on the mRNA expression of P53 (A), Bax (B) and Bcl2 (C) in cardiac tissue. Rats were randomly divided into four different groups of ten animals each: control, CP, probucol and probucol plus CP. Control rats received corn oil for two weeks. Rats in CP group were injected with corn oil for one week before and one week after a single dose of CP (200 mg/kg, I.P.). Rats in group 3 (probucol group) were injected with probucol (61 mg/kg/day, I.P.) for two weeks. Rats in the fourth group (probucol plus CP group) received probucol (61 mg/kg/day, I.P.) for one week before and one week after a single dose of CP (200 mg/kg, I.P.). At the end of the treatment protocol, animals were sacrificed, hearts were isolated and total RNA was extracted for measurement of P53, Bcl2 and Bax genes expression. Data are presented as mean \pm S.E.M. (n=10). *and # indicate significant change from control and CP, respectively, at $p < 0.05$ using ANOVA followed by Tukey–Kramer as a post ANOVA test.

decrease in the antioxidant defense mechanisms in the heart and that antioxidant compounds attenuated CP-induced cardiotoxicity. Interestingly, treatment with probucol completely prevented the increase in serum cardiac enzymes and lipid profile as well as TBARS induced by CP, suggesting that probucol may have potential protective effect against CP-induced cardiac damage. The protective effects of probucol against several forms of cardiomyopathies and congestive heart failure has been previously reported.³³⁻³⁵ Also, lipid-lowering and antioxidant effects of probucol have been previously reported.^{33,36} The contribution of oxidative stress and lipid peroxidation during development of CP-induced cardiotoxicity have been recently reported.^{37,38} Increased oxidative stress biomarkers and depletion of enzymatic and non-enzymatic antioxidants have been reported in cancer patients and other human diseases.^{39,40}

Oxidative stress exerts both agonistic and antagonistic effects on apoptotic signaling through regulation of apoptosis, mediate cell proliferation and differentiation.¹¹ It fractionated the cellular p53 traffics to mitochondria causing p53-dependent cell cycle arrest, activation of Bax in a caspase-dependent manner and initiates apoptosis.¹² Bcl2 regulates apoptosis and acts along intrinsic mitochondrial apoptosis pathway that is activated in response to oxidative stress.¹³ Apoptosis is one of the major processes that lead to the progressive decline of myocardial function responsible for some cardiac pathologies including heart failure, hypertrophy, and myocardial infarction.^{41,42} Signal transduction pathways involved in drug-induced apoptosis congregate on a common pathway that consists of effector molecules, adaptor molecules, and regulatory molecules. The transcription factor p53 has been reported to play a very important role in apoptosis.^{43,44} Many exogenous stimuli, including genotoxic agents, promote the accumulation of the p53 protein in the nucleus, which induces growth arrest and apoptosis.⁴² It has been reported that CP-related cardiomyopathy is linked to its ability to induce apoptosis in myocytes by different mechanisms including DNA intercalation, activation of p53 protein and generation of ROS. Our results showed that CP significantly increases mRNA expression of P53 and Bax and decreases the expression of Bcl2. Interestingly, probucol supplementation completely restored CP-induced upregulation of P53 and Bax genes and downregulation in Bcl2 gene to the normal values, suggesting that probucol may blocks CP-induced apoptosis in

hydrolysis and reducing cholesterol efflux.³⁰ Overproduction of reactive oxygen species (ROS) during CP therapy cause membrane injury by initiating lipid peroxidation that result in loss of function and integrity of myocardial membranes.³¹ This hypothesis is confirmed by the data presented in this study that demonstrated that CP increased TBARS, an index of lipid peroxidation, and decreased GSH, an index of antioxidant defense mechanism, in cardiac tissues. Under similar experimental condition, Machida³² reported that CP (200 mg/kg)-induced acute cardiotoxicity was attributed to the increase in ROS and the

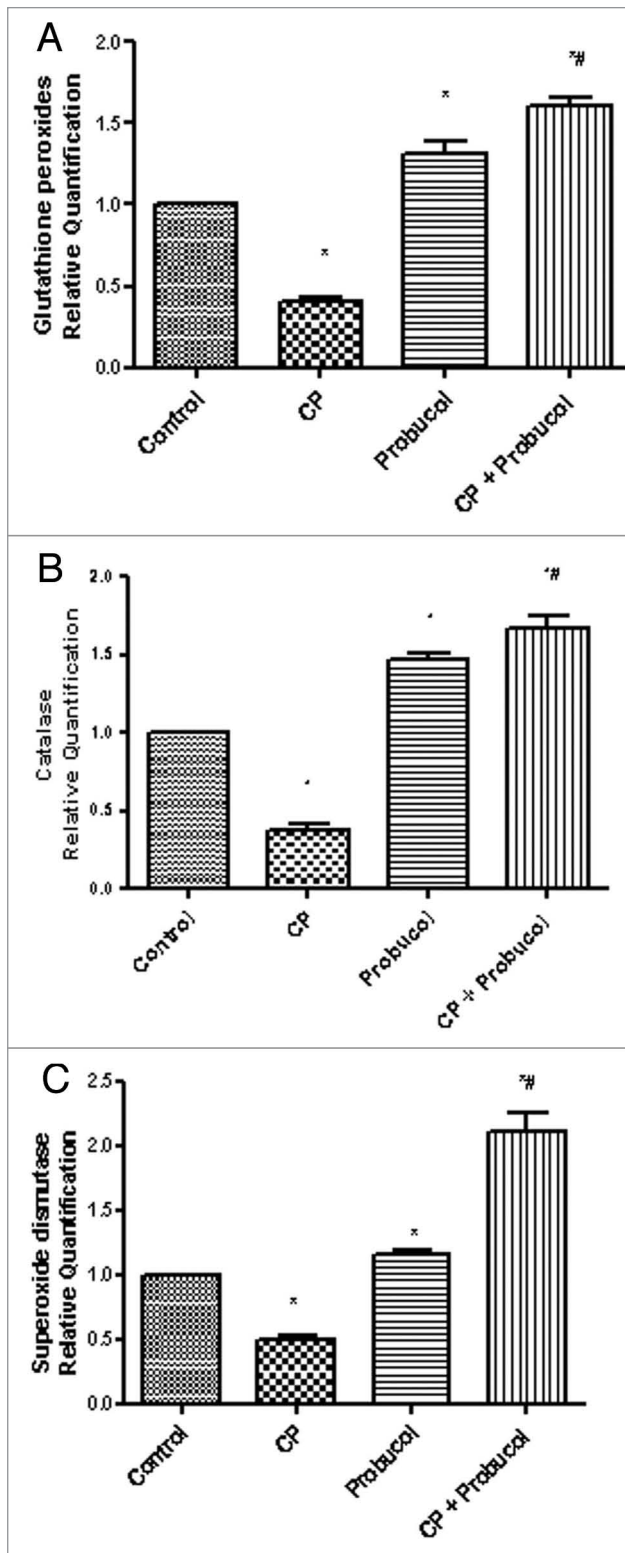


Figure 6. Effect of cyclophosphamide (CP), probucol and cyclophosphamide (CP) plus probucol on the mRNA expression of Glutathione peroxidases (A), Catalase (B) and Superoxide dismutase (C) in cardiac tissue. Rats were randomly divided into four different groups of ten animals each: control, CP, probucol and probucol plus CP. Control rats received corn oil for two weeks. Rats in CP group were injected with corn oil for one week before and one week after a single dose of CP (200 mg/kg, I.P.). Rats in group 3 (probuco group) were injected with probucol (61 mg/kg/day, I.P.) for two weeks. Rats in the fourth group (probuco plus CP group) received probucol (61 mg/kg/day, I.P.) for one week before and one week after a single dose of CP (200 mg/kg, I.P.). At the end of the treatment protocol, animals were sacrificed, hearts were isolated and total RNA was extracted for measurement of Glutathione peroxidases, Catalase and Superoxide dismutase genes expression. Data are presented as mean \pm S.E.M. (n=10). * and # indicate significant change from control and CP, respectively, at $p < 0.05$ using ANOVA followed by Tukey–Kramer as a post ANOVA test.

The antioxidant enzymes glutathione peroxidase, catalase, and superoxide dismutase act in coordination to combat the formed ROS. Decrease in the activities of the antioxidant enzymes in the myocytes of CP administered rats were due to the inactivation of these enzymes by ROS.⁴⁷ This causes further elevation in the levels of ROS, which severely decrease the activities of glutathione peroxidase, catalase, and superoxide dismutase.⁴⁸ The probucol-treated group showed improved activities of glutathione peroxidase, catalase, and superoxide dismutase than the CP group. These evidenced the low ROS level and ROS-mediated inactivation of enzymes were prevented by probucol protecting the myocytes from damage.

In the current study, the observed decrease of ATP and ATP/ADP level in cardiac tissue by CP was parallel to the marked increase in LDH and CK-MB, which may point to the possible consideration of energy starvation as a risk factor in CP-induced cardiotoxicity. Our results are consistent with the data presented by Fatani et al.³⁷ who demonstrated that CP decreased ATP level in cardiac tissues. On the other hand, probucol attenuated CP-induced decline in ATP production in cardiac tissues. Improved energy production by probucol in isoproterenol-induced congestive heart failure has been previously reported.³⁵

Materials and Methods

Animals. Adult male Wistar albino rats, weighing 230-250 g, were obtained from the Animal Care Center, College of Pharmacy, King Saud University (KSU), Riyadh, Kingdom of Saudi Arabia (KSA) and were housed in metabolic cages under controlled environmental conditions (25°C and a 12 h light/dark cycle). Animals had free access to pulverized standard rat pellet food and tap water unless otherwise indicated. The protocol of this study has been approved by Research Ethics Committee of College of Pharmacy, KSU, Riyadh, KSA.

Materials. Endoxan vials (Baxter, Germany) were a gift from King Khalid University Hospital Drug Store, KSU, KSA. Each Endoxan vial contained 500 mg CP in a dry lyophilized powder form. The content of each vial was freshly dissolved in sterile water for injection immediately before injection. Probuco (Sigma Chemical Co., St. Louis, MO) was dissolved in corn oil

cardiac myocytes. These data suggest that CP-induced apoptosis is mediated by oxidative stress and may play a role in the development of heart failure. These observations have been reported previously.⁴⁵ Free radicals play an important role in the mediation of cardiac injury.⁴⁶ The myocardial tissue has the endogenous antioxidant enzymes which protect it from the oxidative damage.

and administered I.P. Intraperitoneal injection was selected because probucol is poorly absorbed from the gastrointestinal tract, with only 2–8% of the dose reaching the circulation.⁴⁹ All other chemicals used were of the highest analytical grade.

Experimental design. In this study, the dose of CP (200 mg/kg, I.P.) used to develop cardiotoxicity has been previously reported.²³

On the other hand, the selected dose of probucol, dissolved in corn oil, (61 mg/kg/day, I.P.) administered for two weeks has been reported to protect against heart failure in rats.^{35,50} A total of 40 male Wistar albino rats were used and divided at random into four groups of ten animals each. Rats of group 1 (control group) received corn oil (2.5 ml/kg/day, I.P.) for two weeks. Rats in group 2 (CP group) were injected with corn oil for one week before and one week after a single dose of CP (200 mg/kg, I.P.). Animals in group 3 (probucol group) were injected with probucol (61 mg/kg/day, I.P.) for two weeks. Animals in the fourth group (probucol plus CP group) received probucol (61 mg/kg/day, I.P.) for one week before and one week after a single dose of CP (200 mg/kg, I.P.). Table 1 outlines the sequence of studies for each experimental animal model used.

At the end of the treatment protocol, rats were anesthetized with ether, and blood samples were obtained by heart puncture. Sera were separated for measurement of triglycerides, cholesterol, lactate dehydrogenase (LDH), and creatine kinase (CK-MB). Animals were then sacrificed by decapitation after exposure to ether in a desiccator kept in a well-functioning hood and hearts were isolated. The hearts were quickly excised, washed with saline, blotted with a piece of filter paper, and homogenized in normal saline using a potter-Elvehjem homogenizer or 6 % perchloric acid as indicated in the procedures of measurement of each parameter.

Measurement of CK-MB and LDH activities in serum. Serum activities of cardiotoxicity indices LDH and CK-MB were determined according to the methods of Buhl and Jackson⁵¹ and Wu and Bowers,⁵² respectively.

Determination of serum cholesterol and triglycerides. Cholesterol and triglycerides, which are used as risk factors in cardiovascular diseases, were determined according to the methods of Parekh and Jung,⁵³ Foster and Dunn,⁵⁴ respectively.

Determination of reduced glutathione and lipid peroxidation in heart tissues. The tissue levels of the acid soluble thiols, mainly GSH as index of antioxidant defense mechanism in cardiac tissues, were assayed spectrophotometrically at 412 nm.⁵⁵ The contents of GSH were expressed as $\mu\text{mol/gm}$ wet tissue. The degree of lipid peroxidation in serum and heart tissues was determined by measuring thiobarbituric acid reactive substances (TBARS) in the supernatant from tissue homogenate.⁵⁶ 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.

Gene expression profile by real time PCR. Total RNA was extracted from heart tissue by Trizol method according to the standard protocol as previously described.⁵⁸ In Briefly, RNA

Table 1. Sequence of studies

Group number	Types and duration of treatments		
	First week	The day at the end of the week	Second week
1		Corn oil	
2	Corn oil	CP	Corn oil
3		probucol	
4	probucol	CP	probucol

was extracted by homogenization (Polytron, Switzerland) in TRIzol reagent (Gibco BRL) at maximum speed for 90–120 s. The homogenate was incubated for 5 min. A 1:5 volume of chloroform was added, and the tube was vortexed and subjected to centrifugation. The aqueous phase was isolated, and one-half of the volume of isopropanol was added to precipitate the RNA. After centrifugation and washing the total RNA was finally eluted in 20 μl of diethyl pyrocarbonate-treated H₂O, and the quantity and integrity were characterized using a UV spectrophotometer.

First-strand cDNA synthesis using SuperScript II RT. First-strand cDNA was synthesized from 1 μg of total RNA by reverse transcription with a SuperScript™ first-strand synthesis system kit (Invitrogen, USA), according to the manufacturer's instructions.

SYBR Green real-time PCR. We used GAPDH as the house-keeping gene. The genes levels were measured using real time PCR with SYBR Green dye and the 2^{- $\Delta\Delta\text{Ct}$} method. The PCR assay was carried out with 25 μl of a real-time PCR mixture consisting of 12.5 μl of 2 \times SYBR Green Supermix (Sigma, USA) and 200 μM primers (each). Next, 2 μl of the cDNA was added to the reaction mixture. The SYBR Green Supermix contained dNTP (0.4 mM), Taq polymerase, 6 mM MgCl₂, 100 mM KCl, and 40 mM Tris-HCl (pH 8.4). The amplification was performed in Applied biosystem (USA). The cycling program consists of 95° C for 10min followed by 40 cycles of denaturation at 94° C for 30s, annealing/extension temperature at 60° C for 1 min. Finally, a melting curve analysis was undertaken from 60° C to 95° C. The real time PCR yields a value (Ct) having the threshold cycle of specific target gene amplification at which the PCR products were first detected via fluorescence.

Melting curve and agarose gel electrophoresis analysis. Following amplification, melting curve analysis was performed to verify the correct product according to its specific melting temperature (T_m). The results were analyzed by the melting curve analysis software of Applied Biosystem. Amplification plots and T_m values were routinely analyzed to confirm the specificities of the amplicons for SYBR Green-based PCR amplification. Agarose gel electrophoresis for detection of GAPDH, P53, Bax, Bcl2, catalase, Glutathione peroxides and Superoxide dismutase. amplification showed the existence of a single band for each gene.

Statistical analysis. Differences between obtained values (mean \pm SEM, $n = 10$) were carried out by one way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test. A p value of 0.05 or less was taken as a criterion for a statistically significant difference.

Table 2. Primers sequence of the GPX, CAT, P53, Bcl2, Bax and GAPDH

Gene name	Forward primer	Reverse primer
GPX	5'- GGGCAAAGAAGATTCCAGTT -'3	5'- AGAGCGGGTGAGCCTTCT -'3
CAT	5'- AGGTGACACTATAGAATAGTGGTTTCACCGACGAGAT -'3	5'- GTACGACTCACTATAGGGACACGAGGTCCAGTTACCAT -'3
SOD	5'-GCAGAAGGCAAGCGGTGAAC-3	5'-TAGCAGGACAGCAGATGAGT'3
P53	5'-CAGCGTGA TGATGTAAGGA-3	5'-GCGTTGCTCTGATGGTGA-3
Bcl2	5'-TCTTCAGGCTGGAAGGAGAA-3	5'-AAGTGTCACAGAGGGGCTA-3
Bax	5'-GATCAGCTCGGGCACTTTAG-3	5'-TGTTTGCTGATGGCAACTTC-3
GAPDH	5'-CCCTTCATTGACCTCAACTACAATGGT-3	5'-GAGGGGCCATCCACAGTCTTCTG-3
FIH HIF	Reverse	GAT TGT CAA AGT CCA CCT GGC T
Notch-1	Forward	AGG ACC TCA TCA ACT CAC ACG C
Notch-1	Reverse	CGT TCT TCA GGA GCA CAA CTG C
Jagged-1	Forward	TGT CTG TCC CAC TGG TTT CTC
Jagged-1	Reverse	AGT TCT TGC CCT CAT AGT CCT CG
HES-1	Forward	CCA AAG ACA GCA TCT GAG CA
HES-1	Reverse	TCA GCT GGC TCA GAC TTT CA

Conclusion

Data from the present study suggest that probucol prevents the development of CP-induced cardiotoxicity by its ability to increase mRNA expression of antioxidant genes and to decrease

apoptosis in cardiac tissues with the consequent improvement in mitochondrial oxidative phosphorylation and energy production. This will open new perspectives for the use of probucol in the treatment of CP-related cardiotoxicity.

References

- Fraiser LH, Kanekal S, Kehrer JP. Cyclophosphamide toxicity. Characterising and avoiding the problem. *Drugs* 1991; 42:781-95.
- Budd GT, Ganapathi R, Wood L, Snyder J, McLain D, Bukowski RM. Approaches to managing carboplatin-induced thrombocytopenia: focus on the role of amifostine. *Semin Oncol* 1999; 26:41-50.
- Sladek NE. Metabolism of oxazaphosphorines. *Pharmacol Ther* 1988; 37:301-55.
- Sladek NE. Metabolism of cyclophosphamide by rat hepatic microsomes. *Cancer Res* 1971; 31:901-8.
- Shanholtz C. Acute life-threatening toxicity of cancer treatment. *Crit Care Clin* 2001; 17:483-502.
- Schimmel KJ, Richel DJ, van den Brink RB, Guchelaar HJ. Cardiotoxicity of cytotoxic drugs. *Cancer Treat Rev* 2004; 30:181-91.
- Louder AM, Dousset N, Carton M, Douste-Blazy L. Effects of an antimetabolic agent (cyclophosphamide) on plasma lipoproteins. *Biochem Pharmacol* 1984; 33:2961-5.
- Lespine A, Chap H, Perret B. Impaired secretion of heart lipoprotein lipase in cyclophosphamide-treated rabbit. *Biochim Biophys Acta* 1997; 1345:77-85.
- Cheng L, Ding G, Qin Q, Huang Y, Lewis W, He N, et al. Cardiomyocyte-restricted peroxisome proliferator-activated receptor-delta deletion perturbs myocardial fatty acid oxidation and leads to cardiomyopathy. *Nat Med* 2004; 10:1245-50.
- Franco R, Sanchez-Olea R, Reyes-Reyes EM, Panayiotidis ML. Environmental toxicity, oxidative stress and apoptosis: manage a trois. *Mutat Res* 2009; 674:3-22.
- Liu B, Chen Y, St Clair DK. ROS and p53: a versatile partnership. *Free Radic Biol Med* 2008; 44:1529-35.
- Chaudhari M, Jayaraj R, Bhaskar AS, Lakshmana Rao PV. Oxidative stress induction by T-2 toxin causes DNA damage and triggers apoptosis via caspase pathway in human cervical cancer cells. *Toxicology* 2009; 262:153-61.
- Frenzel A, Grespi F, Chmielewski W, Villunger A. Bcl2 family proteins in carcinogenesis and the treatment of cancer. *Apoptosis* 2009; 14:584-96.
- Zimetbaum P, Eder H, Frishman W. Probuco: pharmacology and clinical application. *J Clin Pharmacol* 1990; 30:3-9.
- Kaul N, Siveski-Iliskovic N, Hill M, Khaper N, Seneviratne C, Singal PK. Probuco treatment reverses antioxidant and functional deficit in diabetic cardiomyopathy. *Mol Cell Biochem* 1996; 160-161:283-8.
- Siveski-Iliskovic N, Hill M, Chow DA, Singal PK. Probuco protects against adriamycin cardiomyopathy without interfering with its antitumor effect. *Circulation* 1995; 91:10-5.
- Li T, Singal PK. Adriamycin-induced early changes in myocardial antioxidant enzymes and their modulation by probuco. *Circulation* 2000; 102:2105-10.
- Nakamura N, Obayashi H, Fujii M, Fukui M, Yoshimori K, Ogata M, et al. Induction of aldose reductase in cultured human microvascular endothelial cells by advanced glycation end products. *Free Radic Biol Med* 2000; 29:17-25.
- Iqbal M, Sharma SD, Okada S. Probuco as a potent inhibitor of oxygen radical-induced lipid peroxidation and DNA damage: in vitro studies. *Redox Rep* 2004; 9:167-72.
- Gharib MI, Burnett AK. Chemotherapy-induced cardiotoxicity: current practice and prospects of prophylaxis. *Eur J Heart Fail* 2002; 4:235-42.
- Lee CK, Harman GS, Hohl RJ, Gingrich RD. Fatal cyclophosphamide cardiomyopathy: its clinical course and treatment. *Bone Marrow Transplant* 1996; 18:573-7.
- Appelbaum F, Strauchen JA, Graw RG, Jr., Savage DD, Kent KM, Ferrans VJ, Herzig GP. Acute lethal carditis caused by high-dose combination chemotherapy. A unique clinical and pathological entity. *Lancet* 1976; 1:58-62.
- Mythili Y, Sudharsan PT, Selvakumar E, Varalakshmi P. Protective effect of DL-alpha-lipoic acid on cyclophosphamide induced oxidative cardiac injury. *Chem Biol Interact* 2004; 151:13-9.
- Mythili Y, Sudharsan PT, Varalakshmi P. Cytoprotective role of DL-alpha-lipoic acid in cyclophosphamide induced myocardial toxicity. *Mol Cell Biochem* 2005; 276:39-44.
- Sudharsan PT, Mythili Y, Selvakumar E, Varalakshmi P. Lupeol and its ester ameliorate the cyclophosphamide provoked cardiac lysosomal damage studied in rat. *Mol Cell Biochem* 2006; 282:23-9.
- Mythili Y, Sudharsan PT, Amudha G, Varalakshmi P. Effect of DL-alpha-lipoic acid on cyclophosphamide induced lysosomal changes in oxidative cardiotoxicity. *Life Sci* 2007; 80:1993-8.
- Fatani AG, Darweesh AQ, Rizwan L, Aleisa AM, Al-Shabanah OA, Sayed-Ahmed MM. Carnitine deficiency aggravates cyclophosphamide-induced cardiotoxicity in rats. *Chemotherapy*; 56:71-81.
- Takami H, Matsuda H, Tagawa K. Energy metabolism and cell injury in ischemic heart. *Tanpakushitsu Kakusan Koso* 1990; 35:1809-15.
- Mythili Y, Sudharsan PT, Sudhahar V, Varalakshmi P. Protective effect of DL-alpha-lipoic acid on cyclophosphamide induced hyperlipidemic cardiomyopathy. *Eur J Pharmacol* 2006; 543:92-6.
- Gesquiere L, Loreau N, Minnich A, Davignon J, Blache D. Oxidative stress leads to cholesterol accumulation in vascular smooth muscle cells. *Free Radic Biol Med* 1999; 27:134-45.
- Janero DR, Hreniuk D, Sharif HM. Hydrogen peroxide-induced oxidative stress to the mammalian heart-muscle cell (cardiomyocyte): lethal peroxidative membrane injury. *J Cell Physiol* 1991; 149:347-64.
- Machida Y, Kubota T, Kawamura N, Funakoshi H, Ide T, Utsumi H, et al. Overexpression of tumor necrosis factor-alpha increases production of hydroxyl radical in murine myocardium. *Am J Physiol Heart Circ Physiol* 2003; 284:H449-55.
- El-Demerdash E, Ali AA, Sayed-Ahmed MM, Osman AM. New aspects in probuco cardioprotection against doxorubicin-induced cardiotoxicity. *Cancer Chemother Pharmacol* 2003; 52:411-6.
- Simpson C, Herr H, Courville KA. Concurrent therapies that protect against doxorubicin-induced cardiomyopathy. *Clin J Oncol Nurs* 2004; 8:497-501.

35. El-Demerdash E, Awad AS, Taha RM, El-Hady AM, Sayed-Ahmed MM. ProbucoI attenuates oxidative stress and energy decline in isoproterenol-induced heart failure in rat. *Pharmacol Res* 2005; 51:311-8.
36. Miida T, Seino U, Miyazaki O, Hanyu O, Hirayama S, Saito T, et al. ProbucoI markedly reduces HDL phospholipids and elevated prebeta1-HDL without delayed conversion into alpha-migrating HDL: putative role of angiotensin-like protein 3 in probucoI-induced HDL remodeling. *Atherosclerosis* 2008; 200:329-35.
37. Fatani AG, Darweesh AQ, Rizwan L, Aleisa AM, Al-Shabanah OA, Sayed-Ahmed MM. Carnitine deficiency aggravates cyclophosphamide-induced cardiotoxicity in rats. *Chemotherapy* 2010; 56:71-81.
38. Todorova V, Vanderpool D, Blossom S, Nwokedi E, Hennings L, Mrak R, et al. Oral glutamine protects against cyclophosphamide-induced cardiotoxicity in experimental rats through increase of cardiac glutathione. *Nutrition* 2009; 25:812-7.
39. Gupta A, Bhatt ML, Misra MK. Lipid peroxidation and antioxidant status in head and neck squamous cell carcinoma patients. *Oxid Med Cell Longev* 2009; 2:68-72.
40. Fisher-Wellman K, Bell HK, Bloomer RJ. Oxidative stress and antioxidant defense mechanisms linked to exercise during cardiopulmonary and metabolic disorders. *Oxid Med Cell Longev* 2009; 2:43-51.
41. Haunstetter A, Izumo S. Apoptosis: basic mechanisms and implications for cardiovascular disease. *Circ Res* 1998; 82:1111-29.
42. Bromme HJ, Holtz J. Apoptosis in the heart: when and why? *Mol Cell Biochem* 1996; 163-164:261-75.
43. Agarwal ML, Taylor WR, Chernov MV, Chernova OB, Stark GR. The p53 network. *J Biol Chem* 1998; 273:1-4.
44. Chen X, Ko LJ, Jayaraman L, Prives C. p53 levels, functional domains, and DNA damage determine the extent of the apoptotic response of tumor cells. *Genes Dev* 1996; 10:2438-51.
45. Kumar D, Kirshenbaum LA, Li T, Danelisen I, Singal PK. Apoptosis in adriamycin cardiomyopathy and its modulation by probucoI. *Antioxid Redox Signal* 2001; 3:135-45.
46. Hill MF, Palace VP, Kaur K, Kumar D, Khaper N, Singal PK. Reduction in oxidative stress and modulation of heart failure subsequent to myocardial infarction in rats. *Exp Clin Cardiol* 2005; 10:146-53.
47. Selvakumar E, Prahathan C, Mythili Y, Varalakshmi P. Protective effect of DL-alpha-lipoic acid in cyclophosphamide induced oxidative injury in rat testis. *Reprod Toxicol* 2004; 19:163-7.
48. Pigeolet E, Corbisier P, Houbion A, Lambert D, Michiels C, Raes M, et al. Glutathione peroxidase, superoxide dismutase, and catalase inactivation by peroxides and oxygen derived free radicals. *Mech Ageing Dev* 1990; 51:283-97.
49. Yamamoto K, Fukuda N, Shiroi S, Shiotsuki Y, Nagata Y, Tani T, et al. Effects of dietary fat levels on the absorption and tissue accumulation of probucoI in the rat. *Arzneimittelforschung* 1994; 44:1059-62.
50. Sia YT, Lapointe N, Parker TG, Tsoporis JN, Deschepper CF, Calderone A, et al. Beneficial effects of long-term use of the antioxidant probucoI in heart failure in the rat. *Circulation* 2002; 105:2549-55.
51. Buhl SN, Jackson KY. Optimal conditions and comparison of lactate dehydrogenase catalysis of the lactate-to-pyruvate and pyruvate-to-lactate reactions in human serum at 25, 30, and 37 degrees C. *Clin Chem* 1978; 24:828-31.
52. Wu AH, Bowers GN, Jr. Evaluation and comparison of immunoinhibition and immunoprecipitation methods for differentiating MB and BB from macro forms of creatine kinase isoenzymes in patients and healthy individuals. *Clin Chem* 1982; 28:2017-21.
53. Parekh AC, Jung DH. An improved method for determination of total hydroxyproline in urine. *Biochem Med* 1970; 4:446-56.
54. Foster LB, Dunn RT. Stable reagents for determination of serum triglycerides by a colorimetric Hantzsch condensation method. *Clin Chem* 1973; 19:338-40.
55. Davies MH, Birt DF, Schnell RC. Direct enzymatic assay for reduced and oxidized glutathione. *J Pharmacol Methods* 1984; 12:191-4.
56. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979; 95:351-8.
57. Botker HE, Kimose HH, Helligso P, Nielsen TT. Analytical evaluation of high energy phosphate determination by high performance liquid chromatography in myocardial tissue. *J Mol Cell Cardiol* 1994; 26:41-8.
58. Chomczynski P. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *Biotechniques* 1993; 15:532-4, 6-7.