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Probucol attenuates oxidative stress and energy decline in isoproterenol-induced heart failure in rat

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Abstract

In the present study, we examined whether the powerful antioxidant probucol (a clinically used lipid-lowering drug) would attenuate the oxidative stress and energy starvation in experimental model of heart failure (HF) using isoproterenol. Rats were injected subcutaneously with isoproterenol (2.4 mg kg^{-1}) daily for 1 week, and then treated with probucol (61 mg/kg) daily for 2 weeks. Oxidative stress was assessed by measuring myocardial lipid peroxides level and antioxidant enzymes activities, glutathione peroxidase (GPx) and superoxide dismutase. In addition, cardiac metabolic damage was estimated by measuring myocardial ATP, ADP and AMP levels as well as ATP/ADP ratio. It was found that isoproterenol induced a significant increase in heart rate by approximately 30% as compared with the pre-value. These changes were significantly attenuated by post-treatment of rats with probucol. Also, isoproterenol induced several pathological changes including lymphocyte infiltration, myofibrillar hemorrhage and degeneration, and these changes were attenuated by probucol. In addition, animals treated with isoproterenol showed a significant increase in myocardial lipid peroxides level up to 163% and a significant decrease in myocardial GPx activity by 35% as compared with the control group. Probucol not only counteracted significantly the pronounced oxidative stress effect of isoproterenol but also it induced a significant increase in myocardial GPx as compared with the control group. The major new finding of the present study is that treatment with probucol induced a significant increase in myocardial ATP level (the source of energy) and ATP/ADP ratio. Moreover, there is a significant correlation between ATP/ADP ratio and myocardial probucol level. In conclusion, the cardioprotective effect of probucol in treatment of HF is a result of not only its antioxidant properties and an enhancement of endogenous antioxidant reserve (mainly GPx) but also an enhancement of myocardial energy state.

Keywords: Probucol; Isoproterenol; Heart failure; ATP; Oxidative stress

1. Introduction

Heart failure (HF) is the functional definition of a clinical syndrome with a heterogeneous underlying etiology, but has a common manifestation characterized by the "exhaustion of the reserve force of the heart" [1]. In the last decade, several evidences suggested that oxidative stress and inflammation might be involved in the pathogenic processes in HF [2–4]. Accordingly, recent studies tried to ameliorate cardiac hypertrophy, oxidative stress and inflammation in HF by using certain antioxidants like Vitamin E [5], dimethylthiourea [6] and probucol [7,8].

Probucol is a clinically used cholesterol-lowering drug [9]. Beside its antioxidant properties, probucol was shown to protect against diabetes-associated [10] and adriamycin-induced cardiomyopathy [11,12] by enhancing the endogenous antioxidant system including glutathione peroxidase, catalase and superoxide dismutase.

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Regarding HF, Sia et al. [7,8] reported that treatment with probucol improved survival in rats with large myocardial infarction associated with reduced cardiac fibrosis and expression of inflammatory cytokines. Moreover, Nakamura et al. [13] found that treatment with probucol attenuated myocardial inflammatory processes including monocyte infiltration and matrix metaloproteinases activation in a canine model tachycardia-induced HF.

In our previous study, we found that, probucol increased the myocardial ATP/ADP ratio i.e., probucol can enhance the cellular source of energy [14]. Since HF can be considered as a state of energy starvation, due to an imbalance between energy production and use [15], the beneficial effect of probucol in treatment of HF may be related to its effect on myocardial energy state. Indeed, few therapeutic approaches have been aimed to preventing or opposing the consequences of energy imbalance in HF. For example, propionyl-L-carnitine, an antioxidant that represents the prototype of a novel class of therapeutic agents, was found to be able to stimulate substrate oxidation with consequent increase in ATP/ADP ratio and energy production in hypertrophic isolated rat cardiomyocytes [16].

Therefore, the first goal of the present study was to examine whether the powerful antioxidant probucol would attenuate the oxidative stress and progression of HF experimentally induced by isoproterenol in rat. The second goal was to more explore the effect of probucol on myocardial nucleotides (ATP, ADP and AMP) and its important role in treatment of HF. The model of isoproterenol-induced HF is considered as one of the most widely used experimental model for several reasons. The model is characterized by an extraordinary technical simplicity, an excellent reproducibility as well as an acceptable low mortality [17].

2. Materials and methods

2.1. Animals

Investigation was performed on 60 male albino rats weighing 160–200 g. These animals of our institute's own outbreed stock. The animals were housed in a conditioned atmosphere and kept on a standard diet and water ad libitum.

2.2. Drugs

Isoproterenol (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in saline and injected in dose of 2.4 mg kg^{-1}, subcutaneous (s.c.) as described previously [18]. Probucol (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in corn oil and administered intraperitoneal (i.p.) in dose of 61 mg kg^{-1} according to the study of Sia et al. [8]. Intraperitoneal injection was selected because probucol is poorly absorbed from the gastrointestinal tract, with only 2–8% of the dose reaching the circulation [19]. Animal treatment protocol has been approved by Ethical & Animal Care Committees of National Cancer Institute, Cairo University before starting the experiments.

2.3. Experimental protocol

Sixty male albino rats were divided into four groups ($n = 15$ per group). The rats of groups 1 and 2 were given isoproterenol (2.4 mg kg^{-1}, s.c.) daily for a week, while the other two groups (3 and 4) were given saline. Then, rats of groups 1 and 3 were given probucol (61 mg kg^{-1}, i.p.) daily for 2 weeks, while the other two groups were given corn oil.

Heart rate (HR) was recorded at the beginning of the experiment (before drug injection) and after completion of isoproterenol treatment (1 week) and probucol treatment (3 weeks). At the end of the experiment, all rats were sacrificed. The heart was quickly excised, placed immediately in ice-cold saline to wash it free from blood and weighed. The atria were trimmed away, and the left and right ventricles separated and weighed. Specimens from hearts of each group were fixed in 8% buffered formalin for histopathologic examination. Then seven hearts from each group were homogenized in 0.1 M Tris–HCl buffer (pH 7.4) using a Branson Sonifier (250, VWR Scientific, Danbury, USA). Heart homogenates were used for the measurement of lipid peroxides and protein levels as well as antioxidant enzymes activities including glutathione peroxidase (GPx) and superoxide dismutase (SOD). The rest of hearts ($n = 6$–8) from each group were used for the assessment of myocardial nucleotides (ATP, ADP and AMP) and probucol levels using high performance liquid chromatography (HPLC).

2.3.1. HR measurements

All animals were anaesthetized with ether, needle electrodes were inserted under the skin for the limb and heart rate (expressed in beats/min) was measured using an electrocardiograph (400MD2C, Bioscience, Sheerness, Kent, UK).

2.3.2. Assessment of oxidative stress

Myocardial lipid peroxides was measured according to the method of Ohkawa et al. [20] and protein according to the method of Lowry et al. [21]. Also, the myocardial antioxidant enzyme activities, GPx and SOD were assessed by the methods of Paglia and Valentaine [22] and Marklund [23], respectively.

2.3.3. Assessment of nucleotides and probucol levels

Myocardial nucleotides were determined according to the method of Neri et al. [24]. In brief, part of the hearts was homogenized in 6% perchloric acid using a Branson Sonifier. The clear supernatant was then neutralized by potassium hydroxide and used for determination of ATP, ADP and AMP by using HPLC (Kontron, 322, Sebai, Italy), C18 hypersil column and UV detector at 254 nm. Myocardial probucol level was measured according to the method of Heeg et al. [25]. Briefly, the rest part of the hearts was homogenized (20%)

Table 1
The effect of isoproterenol and probucol treatment on heart rate[a]

Groups (N = 13–15)	Heart rate (beats/min)		
	Pre-value	After 1 week	After 3 weeks
Control	383.3 ± 8.03	373.3 ± 9.89	373.3 ± 6.67
Isoproterenol	373.3 ± 8.90	483.3 ± 9.54[b]	480.0 ± 11.55[b]
Isoproterenol + probucol	380.0 ± 8.94	476.7 ± 12.02[b]	360.0 ± 15.49
Probucol	373.3 ± 7.89	383.3 ± 8.03	376.7 ± 10.85

N: number of animals per group.
[a] Heart rate was recorded at the beginning of the experiment (pre-value) and after 1 and 3 weeks. Each value was expressed as mean ± S.E.M.
[b] Significant difference from pre-value at $P < 0.05$ using ANOVA followed by Dunnet test for post-hoc analysis.

in ethanol–acetone (3:2, v/v). One milliliter aliquot was transferred to a screw-cap test tube and 1 ml of hexane and 0.5 ml of water was added. After mixing and centrifugation, the hexane layer was evaporated to dryness with a nitrogen gas stream and the residue was reconstituted in acetonitrile. Serial dilutions of standard probucol were prepared in acetonitrile for construction of standard curve. The samples were analyzed by HPLC using a Hypersil ODS column with a mobile phase consisting of acetonitrile–water (96:4, w/v). Detection was carried out using ultraviolet absorption at 241 nm.

2.4. Statistical analysis

Data are presented as mean ± S.E.M. Multiple group comparisons were carried out using one way analysis of variance (ANOVA) followed by Tukey–Kramer or Dunnet test for post-hoc analysis. Statistical significance was acceptable to a level of $P < 0.05$. Data analysis was achieved using software program Graphpad instat.

3. Results

3.1. HR and heart weight measurements

During the course of treatment three deaths occurred (two rats receiving isoproterenol and one rat receiving isoproterenol and probucol). In addition, treatment of rats with isoproterenol induced a significant increase in HR by approximately 30% as compared with the pre-value (Table 1). Also, isoproterenol-treated rats exhibited significantly greater absolute and relative heart weights as well as left ventricular weight as compared with the control group (Table 2). The HR changes were significantly attenuated by post-treatment of rats with probucol. After 3 weeks, probucol treatment restored the normal HR (Table 1). Also, rats treated with isoproterenol and probucol showed non-significant differences in absolute and relative heart weights as compared with the control group (Table 2). In addition, animals treated with probucol alone did not show any significant change in all studied parameters as compared with the control values (Tables 1 and 2).

3.2. Assessment of myocardial oxidative stress

After 3 weeks, animals treated with isoproterenol showed a significant increase in myocardial lipid peroxides level up to 163% and a significant decrease in myocardial GPx activity by 35% as compared with the control group. However, there is a non-significant decrease in myocardial SOD activity (88%) as compared with the control group (Table 3). On the other hand, treatment of animals with probucol alone did not show any significant change in myocardial lipid peroxides level as well as SOD activity. However, probucol treatment alone induced a significant increase in myocardial GPx activity by 36% as compared with the control group (Table 3). The pronounced oxidative stress effect of isoproterenol is significantly attenuated by treatment of animals with probucol. The myocardial lipid peroxides level was normalized. Moreover, probucol not only counteracted the effect of isoproterenol on myocardial GPx activity but also it induced a significant increase by 51% as compared with the control group (Table 3).

3.3. Assessment of myocardial nucleotides (ATP, ADP and AMP) and probucol levels

After 3 weeks, rats treated with isoproterenol showed a significant increase in myocardial ADP and AMP up to 193 and 179%, respectively, as compared to the control values. Meanwhile, calculation of ATP/ADP ratio revealed that animals treated with isoproterenol showed a significant decrease in ATP/ADP ratio by 23% as compared with the control value (Fig. 1). On the other hand, probucol treatment alone induced a significant increase not only in the levels of all myocardial nucleotides but also ATP/ADP ratio. The extent of

Table 2
The effect of isoproterenol and/or probucol treatment on heart weight

Groups (N = 13–15)	Absolute heart weight (g)	Relative heart weight[c] (%)	RV wt[d] (g)	LV wt[d] (g)
Control	0.64[b] ± 0.008	0.34[b] ± 0.007	0.26[b] ± 0.013	0.35[b] ± 0.008
Isoproterenol	0.77[a] ± 0.027	0.41[a] ± 0.012	0.34[a] ± 0.031	0.44[a] ± 0.023
Isoproterenol + probucol	0.64[b] ± 0.024	0.35[b] ± 0.012	0.30[b] ± 0.020	0.36[b] ± 0.019
Probucol	0.63[b] ± 0.022	0.35[b] ± 0.010	0.27[b] ± 0.012	0.34[b] ± 0.013

N: number of animals per group. Each value was expressed as mean ± S.E.M. [a,b]Significant difference from control or isoproterenol group, respectively, at $P < 0.05$ using ANOVA followed by Tukey–Kramer test for post-hoc analysis.
[c] Relative heart weight is the weight of the heart/animal body weight × 100.
[d] RV wt, right ventricular weight; LV wt, left ventricular weight.

Table 3
Effects of isoproterenol and/or probucol on myocardial lipid peroxides level and antioxidant enzyme activities

Groups ($N = 7$)	Myocardial lipid peroxides (nmol/g tissue)	Myocardial antioxidant enzymes	
		GPx (U/mg protein)	SOD (U/mg protein)
Control	$43.82^{b} \pm 3.43$	$48.55^{b} \pm 3.93$	36.11 ± 2.75
Isoproterenol	$71.41^{a} \pm 6.67$	$31.45^{a} \pm 2.52$	31.87 ± 2.92
Isoproterenol + probucol	$47.88^{b} \pm 3.79$	$73.38^{a,b} \pm 5.79$	43.18 ± 3.71
Probucol	$46.13^{b} \pm 3.84$	$66.03^{a,b} \pm 4.13$	$47.93^{b} \pm 3.2$

N: number of animals per group. GPx, glutathione peroxidase; SOD, superoxide dismutase, one unit of enzyme activity is defined as the amount of enzyme leading to the oxidation of 1 nmol of NADPH min^{-1} at 37 °C (for GPx) or 50% inhibition of pyrogallol auto-oxidation at room temperature and pH 7.8 (for SOD). Values are presented as mean ± S.E.M. ^{a,b}Significant difference from control or isoproterenol group, respectively, at $P < 0.05$ using ANOVA followed by Tukey–Kramer test for post-hoc analysis.

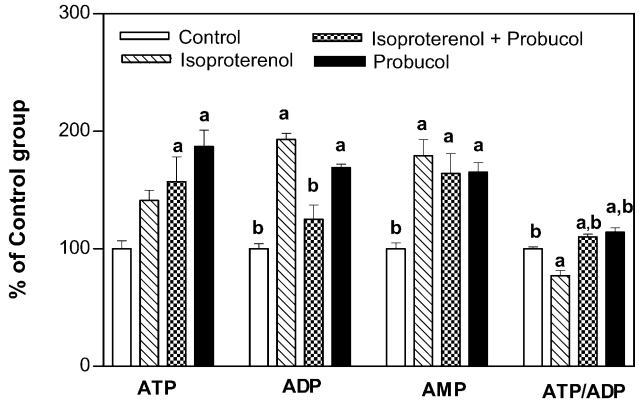

Fig. 1. Comparison of myocardial nucleotides (ATP, ADP and AMP) and ATP/ADP ratio between different groups. Number of samples is eight for control group, six for isoproterenol group, seven for isoproterenol + probucol group and eight for probucol group. a: Significant difference from control group; b: significant difference from isoproterenol group.

increase in myocardial ATP, ADP and AMP levels reached approximately 187, 169 and 165%, respectively and thus, ATP/ADP ratio increased by nearly 14%, as compared to the control value (Fig. 1). Regarding HF treated animals, probucol not only counteracted the decrease in ATP/ADP ratio induced by isoproterenol, but also induced a significant increase by approximately 10% as compared with the control value (Fig. 1). Moreover, a significant correlation was observed between myocardial probucol level and ATP/ADP ratio (Fig. 2).

3.4. Histological examination of cardiac tissue

Hematoxylin–eosin staining was used to evaluate the extent of myocardial inflammation and necrosis. Isoproterenol induced myocardial inflammatory cell infiltration. Lymphocyte infiltrates ranged from isolated and focal in some areas to confluent in others. Also isoproterenol induced myofibrillar hemorrhage, degeneration and fibrosis. Heart specimens from rats treated with isproterenol and probucol showed a minimal degree of cellular hemorrhage and degeneration. In addition, treatment of rats with probucol only did not show any pathological changes (Fig. 3).

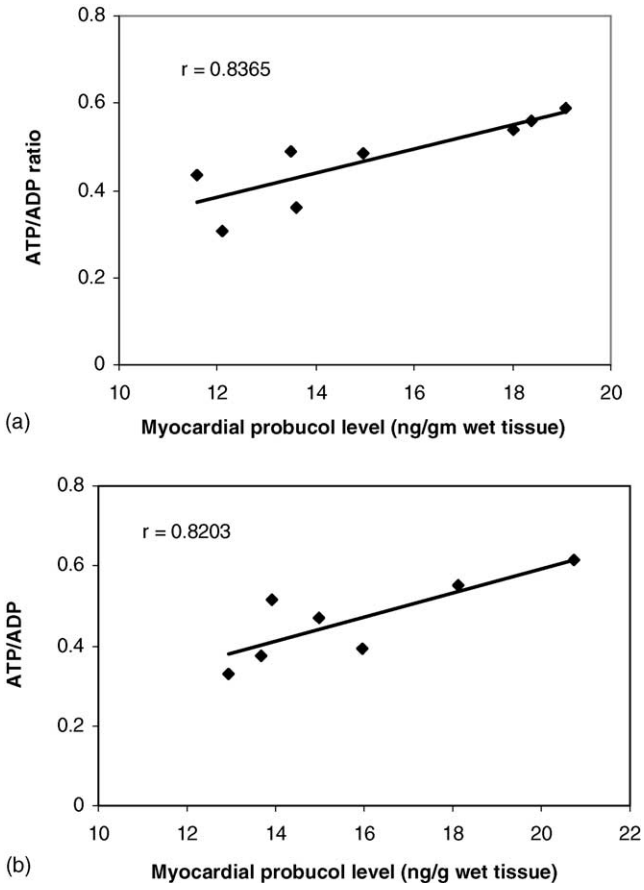

Fig. 2. Correlation between myocardial probucol level and ATP/ADP ratio. (A): Rats treated with probucol ($n = 8$, $P = 0.0096$), (B): rats treated with isoproterenol and probucol ($n = 7$, $P = 0.0238$).

4. Discussion

Heart failure is a serious clinical syndrome with progressive myocardial dysfunction and a poor clinical outcome. Several studies confirmed the role of inflammation and oxidative stress in progression of HF [2–4] and thus attention has been focused on therapies designed to interfere with inflammation and oxidative stress [5–8]. In the present study, we examined whether the powerful antioxidant probucol would attenuate the oxidative stress and the progression of HF

induced experimentally using isoproterenol in rats. Meanwhile, we found previously [14] that probucol enhanced the level of ATP/ADP ratio i.e., it enhanced the cellular source of energy. So, the second goal was to more explore the effect of probucol on myocardial nucleotides (ATP, ADP and AMP) and its important role in treatment of HF, especially that HF can be considered as a state of energy starvation [15].

The present study revealed that animals treated with isoproterenol (2.4 mg/kg, daily for 1 week) showed a significant increase in HR by approximately 30% as compared with the pre-value. In addition, several pathological changes were observed, which are in accordance with previous studies [18,26]. Indeed, the ability of catecholamines, when administered in supraphysiological dosages, to induce infarct like lesion and morphological alterations in the heart resembling HF was already noted early in the last century. However, the exact mechanism of isoproterenol-induced myocardial damage has not been clarified, but a mismatch of oxygen supply versus demand following coronay hypotension and myocardial hyperactivity may offer the best explanation [27]. Other postulated mechanisms including myocardial necrosis and apoptosis as well as free radical generation may contribute in the pathogenesis of catecholamine-induced HF [28]. It has been proposed that, oxidative metabolisms of catecholamines produce quinones, which react with oxygen to produce superoxide anions and hydrogen peroxides [29].

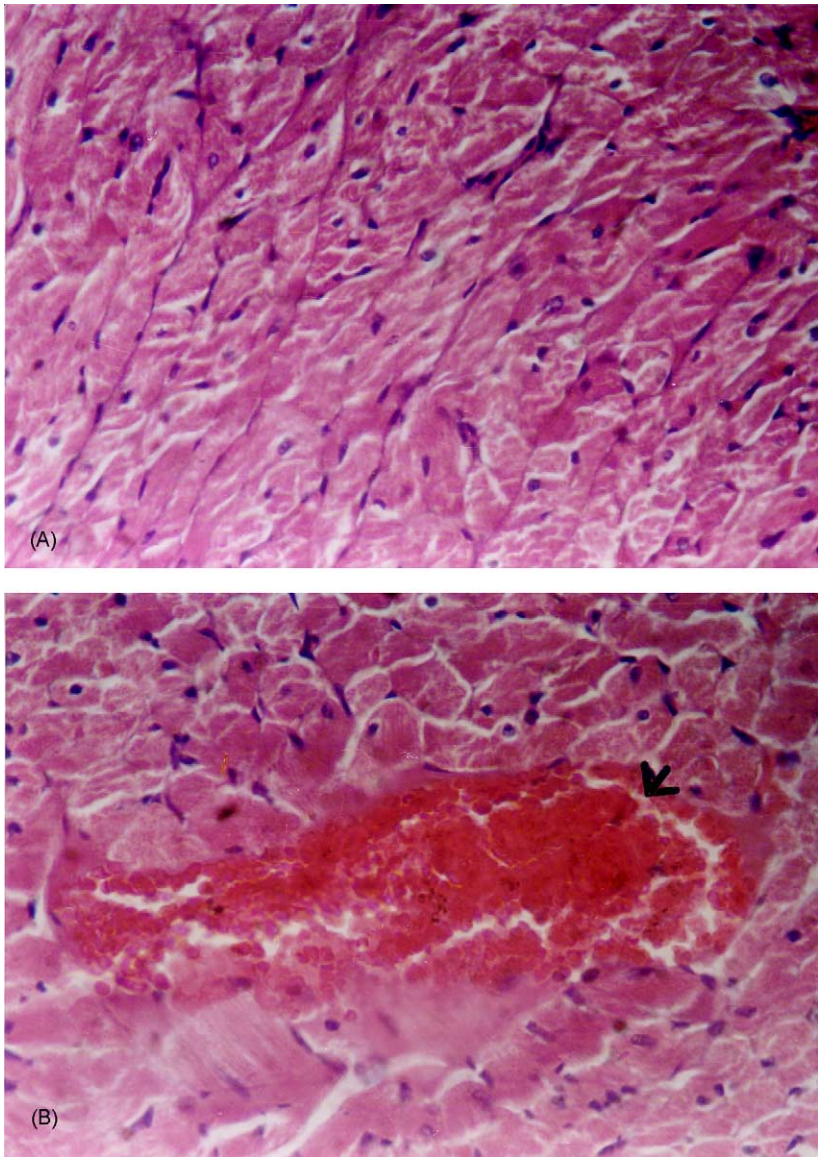

Fig. 3. A photomicrograph of a section in cardiac muscle (hematoxylin–eosin stain, original magnification ×400): (A) represents myocardium of rat treated with corn oil and shows normal cardiac muscle fiber branches and anastomoses with central nucleus. (B and C) Represent myocardium of rat treated with isoproterenol and hemorrhage between cardiac muscle fibers as shown in slide (B), and lymphocyte infiltration and degeneration in cardiac muscle fibers and nuclei shown in slide (C). (D) Represents myocardium of rat treated with isoproterenol and probucol and shows slight hemorrhage and degeneration between few cardiac muscle fibers.

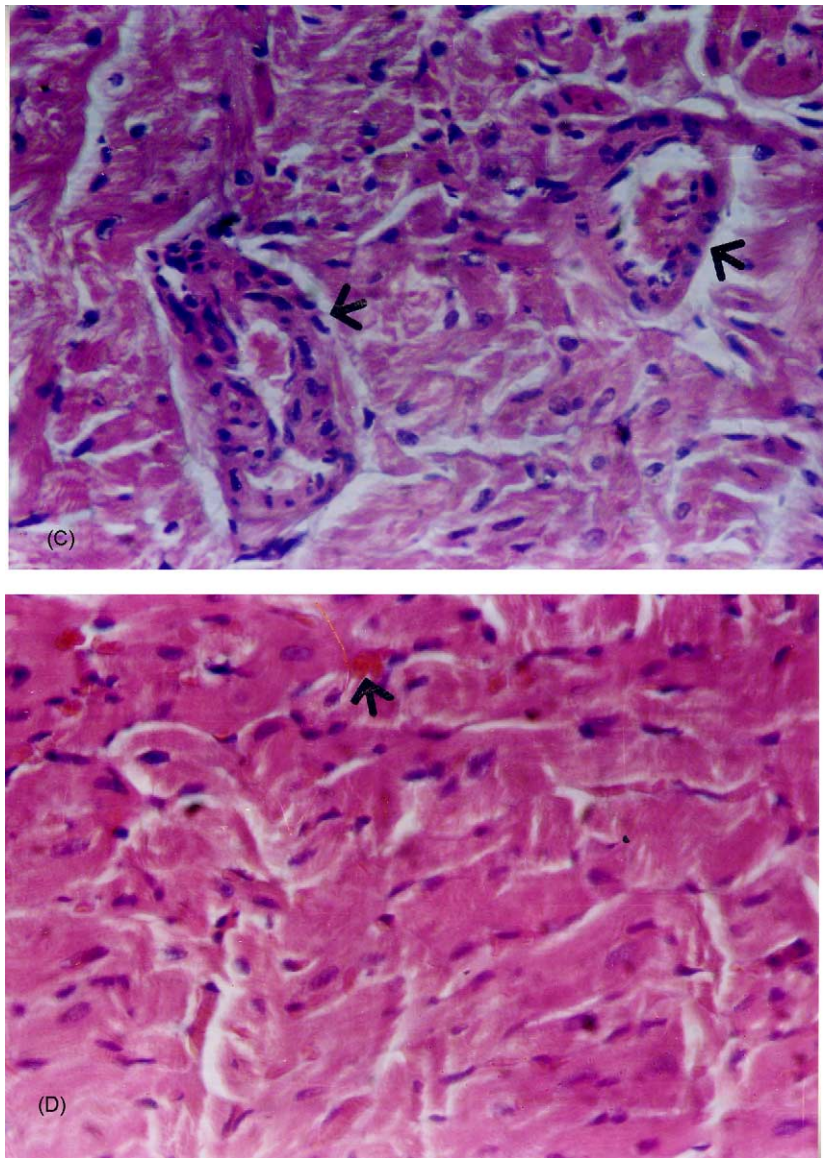

Fig. 3. (*Continued*).

The changes in HR and heart weights as well as the pathological features induced by isoproterenol were found to be significantly attenuated by treatment of rats with probucol indicating its beneficial cardioprotective effect. To explore whether this cardioprotective effect of probucol is related to its antioxidant properties, we assessed myocardial antioxidant enzymes activities (GPx and SOD) as well as lipid peroxides level. After 3 weeks, animals treated with isoproterenol showed a significant increase in myocardial lipid peroxides and a significant decrease in GPx activity as compared with the control group. These findings indicated a state of oxidative stress. However, there is no significant decrease in myocardial SOD activity. Our results are in agreement with previously reported studies [30,31] except the results of myocardial SOD activity. While Rathore et al. [31] reported a significant decrease in myocardial SOD activity; Nirmala and Puvanakrishnan [30] reported a significant increase in enzyme activity. This discrepancy in the results of different studies may be related to the dose and the schedule of isoproterenol injection as well as the time of measurement of enzyme activity.

In addition, we found that the pronounced oxidative stress effect of isoproterenol was significantly reduced by treatment of animals with probucol. Moreover, probucol induced a significant increase in GPx activity as compared with the control group. These findings proved that probucol is not only an antioxidant but it also clearly improves “endogenous antioxidant reserve”, mainly GPx. The enhancement effect of probucol on myocardial GPx has been reported in several previous studies that investigated the effects of probucol against diabetes-associated [10] and adriamycin-induced cardiomyopathy [11,12]. However, these previous studies also showed

that probucol enhanced the endogenous SOD activity, a finding that was not observed in our study.

HF can be considered as a state of energy starvation, due to an imbalance between energy production and use [15]. The energy needed by cardiac muscle to maintain proper function is dependent on a constant resynthesis of ATP by oxidative phosphorylation in the mitochondria. Usually, studies of myocardial energy metabolism examine the myocardial steady state levels of high- and low-energy phosphate that is defined by the ratio of ATP/ADP. In HF, significant alterations in myocardial concentrations of ATP and ADP have been reported [32–34]. In the present study, isoproterenol induced a significant increase in myocardial ADP and AMP levels with a significant decrease in ATP/ADP ratio. These significant changes indicate a state of energy starvation. Chagoya de Sanchez et al. [35] have previously approved this state of energy imbalance.

The important new finding of the present study is that treatment with probucol not only counteracted the effect of isoproterenol but also induced a significant increase in ATP/ADP ratio as compared with the control group. Moreover, we found a significant correlation between the level of probucol in the heart and ATP/ADP ratio. Thus, the cardioprotective effect of probucol can be explained not only by its antioxidant properties but also by enhancing the cellular source of energy.

It is important to note that, Sia et al. in two recent studies [7,8] found that probucol attenuated the progression of left ventricular dysfunction and remodeling (dilatation) and improved the post-myocardial infarction survival. They explained these beneficial pharmacological effects according to the detected anti-inflammatory action of probucol. However, the enhancing effect of probucol on myocardial ATP and ATP/ADP ratio (as proved in our study) may explain more clearly its cardioprotective effect in these previous studies.

In conclusion, the cardioprotective effect of probucol in treatment of HF is a result of not only the inhibition of myocardial oxidative stress and the enhancement of endogenous antioxidant reserve (mainly GPx), but also the increase of ATP (the cellular source of energy). Further studies are needed to explore the mechanistic pathway by which probucol stimulates the ATP synthesis.

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