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Diosmin Prevents Isoproterenol-Induced Heart Mitochondrial Oxidative Stress in Rats

S. Sharmila Queenthy[1,2,3] · P. Stanely Mainzen Prince[4] · Babu John[2]

Abstract Cardiac mitochondrial oxidative stress causes mitochondrial damage that plays an important role in the pathology of myocardial infarction. The preventive effects of diosmin on cardiac mitochondrial oxidative stress in isoproterenol-induced myocardial infarcted rats were evaluated. Rats were pretreated with diosmin (10 mg/kg body weight) daily for 10 days. Myocardial infarction was induced in rats by isoproterenol (100 mg/kg body weight) injection twice at an interval of 24 h (on 11th and 12th day). Isoproterenol-induced myocardial infarcted rats showed a significant increase in the levels of cardiac diagnostic markers, heart mitochondrial lipid peroxidation, calcium ion, and a significant decrease in the levels of heart mitochondrial glutathione peroxidase, reduced glutathione, glutathione-S-transferase, isocitrate, malate, α-ketoglutarate, and succinate dehydrogenases. Transmission electron microscopic findings revealed damaged mitochondria with loss of cristae, swelling, and vacuolation in isoproterenol-induced rats' heart. Diosmin pretreatment showed significant preventive effects on all the biochemical parameters, and the structure of mitochondria was evaluated. Furthermore, the transmission electron microscopic study confirms the biochemical findings. The antioxidant and negative inotropic effects of diosmin inhibited cardiac mitochondrial oxidative stress and prevented mitochondrial damage in myocardial infarcted rats.

Keywords Cardiac markers · Antioxidants · Diosmin · Isoproterenol · Myocardial infarction · Oxidative stress

Introduction

Cardiovascular diseases are a heterogeneous group of disorders that affect the heart and blood vessels. Myocardial infarction is a disease of worldwide significance and increasing prevalence. Reduction in mortality rate and prevention of myocardial infarction are of utmost importance. Myocardial infarction is a death of a segment of heart muscle, which follows interruption of its blood supply. During myocardial infarction, there is huge generation of various reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, and hydroxyl radicals [1]. To understand the prevention, diagnosis, and management of human myocardial infarction, animal models of myocardial infarction are essential [2]. Isoproterenol, a β-adrenergic agonist, induces myocardial infarction when used in excessive dose in the rats [3]. It can produce infarct-like necrosis in the myocardium by severe stress. Oxidative stress is one of the main mechanisms through which isoproterenol exerts its cardiotoxic effects. Isoproterenol-induced myocardial infarction is associated with the generation of ROS, causing oxidative damage in the heart. An increase in the production of free radicals and a decrease in the antioxidant system in the heart are one of the vital risk factors in the occurrence of isoproterenol-

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induced myocardial infarction [4]. With the help of animal models, one can understand the biochemical, molecular, histological, and electrocardiographic changes occurring in human myocardial infarction [5].

The main source of cellular ROS is mitochondria. Oxidative phosphorylation takes place in mitochondria. Further, mitochondria are the main source of energy. During myocardial infarction, oxygen supply is limited. Hence, mitochondrial energy production is impaired. For the cardiac contraction and relaxation, adenosine triphosphate synthesis and electron transport chain which takes place in mitochondria are essential. Mitochondrial damage in myocardial infarction due to oxidative stress causes excessive ROS production, cellular injury, and dysfunction of mitochondria, thereby affecting cardiac contraction and relaxation. Thus, mitochondria are the source and target of ROS-mediated cardiac injury in myocardial infarction [6].

Currently, there has been an increased interest globally to identify antioxidant compounds that are pharmacologically potent and have low or no adverse effects for use in the preventive medicine. Further, consumption of diets rich in flavonoids is associated with reduced risk of cardiovascular diseases such as myocardial infarction. Flavonoids are pharmacologically active and are present in fruits and various plants. They prevent arteriosclerosis, hepatic injury and cancer, by its potent antioxidant activity [7]. Diosmin (3′,5,7-trihydroxy-4′-methoxyflavone 7-rutinoside), an unsaturated flavone, is abundantly present in Hyssop and Rosemary [8]. It has antiinflammatory and antimutagenic properties [9]. Further, certain flavonoids intake can lessen the chances of getting cancer, diabetes mellitus, and cardiovascular diseases because of their strong antioxidant action.

The role of oxidative stress is well established in the progression of human and experimental myocardial infarction. Antioxidant therapy can inhibit oxidative stress and prevent myocardial infarction. Also, preserving cardiac mitochondrial function is one of the best therapeutic approaches to prevent myocardial infarction [10]. Previously, we observed the antihyperlipidaemic effects of diosmin in isoproterenol-induced myocardial infarcted rats [8]. Based on the antihyperlipidaemic effects of diosmin, we hypothesized that diosmin can prevent oxidative stress in mitochondria. In this context, the preventive effects of diosmin on altered lipid peroxidation, antioxidant system, tricarboxylic acid (TCA) cycle enzymes, calcium ions (Ca^{2+}), and structure of mitochondria in isoproterenol-induced myocardial infarcted rats heart were evaluated. Further, to know the mechanism of action of diosmin, in vitro ferric reducing antioxidant power (FRAP) assay was done.

Materials and Methods

Chemicals, Experimental Animals, and Diet

Diosmin, isoproterenol hydrochloride, phenazine methosulphate, and glutathione were purchased from Sigma Chemical Co., St. Louis, MO, USA. All other chemicals and solvents used were of analytical grade. Healthy male albino Wistar rats (*Rattus norvegicus*) weighing 180–200 g were used for all the experiments. They were purchased from Mahaveer Enterprises, Hyderabad, India. The rats were housed in polypropylene cages (47 × 34 × 20 cm) (3 rats/cage) lined with husk, renewed every 24 h under a 12-h light/dark cycle at around 22 °C with 50% humidity. The rats had free access to water and food and fed on a standard pellet diet (Pranav Agro Industries Ltd., Pune, Maharashtra, India). The experiment was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, New Delhi, India, and approved by the Institutional Animal Ethical Committee of Jayamukhi College of Pharmacy (Approval No. 2, Dated; 10/06/2011).

Induction of Experimental Myocardial Infarction and Experimental Design

Isoproterenol hydrochloride (100 mg/kg body weight) dissolved in saline was injected subcutaneously into rats twice at an interval of 24 h [8, 11]. Myocardial infarcted rats were confirmed by increased levels of serum lactate dehydrogenase and myoglobin. The experiment was performed with four groups of rats, each group consisting of six rats. Group-I: Normal control rats were given 2 ml of saline orally by gastric intubation daily for a period of 10 days; Group-II: Rats were treated with diosmin (10 mg/kg body weight) dissolved in 2 ml of saline orally by gastric intubation daily for 10 days; Group-III: Rats were given 2 ml of saline orally by gastric intubation daily for 10 days and then injected subcutaneously with isoproterenol (100 mg/kg body weight) in 2 ml of saline at an interval of 24 h for 2 days (on 11th and 12th day); Group-IV: Rats were pretreated with diosmin (10 mg/kg body weight) dissolved in 2 ml of saline orally by gastric intubation daily for 10 days and then injected subcutaneously with isoproterenol (100 mg/kg body weight) at an interval of 24 h for 2 days (on 11th and 12th day) [8].

At the end of experimental period, after 12 h of second dose of isoproterenol injection (i.e., on 13th day), all the rats were anesthetized with ketamine hydrochloride (100 mg/kg body weight) and then killed by cervical decapitation. Blood was collected in dry test tubes without anticoagulant for serum. The heart was dissected out

immediately, and the infarcted region was separated out and stored for mitochondrial isolation. A portion of the infarcted heart was also used for transmission electron microscopic study. All the enzyme assays were performed on 13th day.

Assay/Estimation of Serum Lactate Dehydrogenase and Myoglobin

The assay of serum lactate dehydrogenase activity was performed by a commercial kit (Agappe Diagnostics, Kerala, India). The serum myoglobin level was estimated by using VITROS immunodiagnostic kit (Ortho-Clinical Diagnostics, Inc. New York, USA).

Isolation of Heart Mitochondria, Estimation/Assay of Lipid Peroxidation Products, and Antioxidants in the Heart Mitochondrial Fraction

Takasawa et al. [12] method was followed for the isolation of mitochondrial fraction from the heart tissue. The heart tissue was put into ice-cold 50 mM Tris–HCl (pH 7.4) containing 0.25 M sucrose and homogenized. The homogenates were centrifuged at $700 \times g$ for 20 min, and the supernatants obtained were centrifuged at $9000 \times g$ for 15 min. Then, the pellets were washed with 10 mM Tris–HCl (pH 7.8) containing 0.25 M sucrose and finally resuspended in the same buffer. The levels/activities of thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD), glutathione peroxidase (GPx), reduced glutathione (GSH), and glutathione-S-transferase (GST) in the heart mitochondria were estimated/assayed by standard procedures [13–17].

Assay/Estimation of Heart Mitochondrial TCA Cycle Enzymes, Ca^{2+} and Protein

The activities of heart mitochondrial TCA cycle enzymes such as isocitrate dehydrogenase (ICDH), malate dehydrogenase (MDH), α-ketoglutarate dehydrogenase (α-KDH) and succinate dehydrogenase (SDH) were assayed by standard methods [18–21]. Heart mitochondrial Ca^{2+} was measured by using a reagent kit (Span Diagnostics Limited, India). The protein content in the heart homogenate was also estimated [22].

Transmission Electron Microscopic Study on the Structure of Heart Mitochondria

Small pieces of heart were taken from normal and experimental rats on 13th day and rinsed in 0.1 M phosphate buffer (pH 7.2). Approximately, 1-mm heart pieces were trimmed and fixed into 3% ice-cold glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) and kept at 4 °C for 12 h. Then, processing of tissue was carried out. The grids containing sections were stained with 2% uranyl acetate and 0.2% lead acetate. Then the sections were examined under a transmission electron microscope (20,000×).

The In Vitro Reducing Activity of Diosmin (FRAP Assay)

The FRAP assay of diosmin was performed according to the method of Benzie and Strain [23]. FRAP reagent was prepared fresh and warmed at 37 °C in a water bath. This reagent is a mixture of 10 mM of 2,4,6-tripyridyl-S-triazine in 40 mM hydrochloric acid, 20 mM ferric chloride, and 0.3 M acetate buffer (pH 3.6) in the ratio of 1:1:10. An aliquot of 25 μl of diosmin was added to 475 μl of FRAP reagent and incubated at 37 °C for 30 min. The absorbance was monitored for various concentrations of diosmin (10, 20, 30, 40 and 50 μM) at 593 nm using a UV–visible spectrophotometer.

Statistical Analysis

Statistical analysis was performed by one-way analysis of variance followed by Duncan's multiple range test using Statistical Package for the Social Science software package version 12.00. Results were expressed as mean ± SD for 6 rats in each group. P values <0.05 were considered significant [8].

Results

Effect of Diosmin on the Activity/Level of Serum Lactate Dehydrogenase and Myoglobin

Isoproterenol-induced myocardial infarcted rats showed a significant ($P < 0.05$) increase in the activity/level of serum lactate dehydrogenase and myoglobin compared to normal control rats. Diosmin (10 mg/kg body weight) pretreatment daily for a period of 10 days significantly ($P < 0.05$) decreased the activity/level of serum lactate dehydrogenase and myoglobin in isoproterenol-induced myocardial infarcted rats compared to isoproterenol-alone-induced myocardial infarcted rats (Fig. 1a, b).

Effect of Diosmin on the Level of TBARS in the Heart Mitochondria

Isoproterenol-induced myocardial infarcted rats revealed a significant ($P < 0.05$) increase in the level of TBARS in

Fig. 1 a, b Activity/level of serum lactate dehydrogenase and myoglobin. *Each column* is the mean ± standard deviation; $n = 6$; columns sharing a common letter *(a, a)* do not differ significantly with each other; columns not sharing a common letter *(a–c)* differ significantly with each other ($P < 0.05$, Duncan's multiple range test)
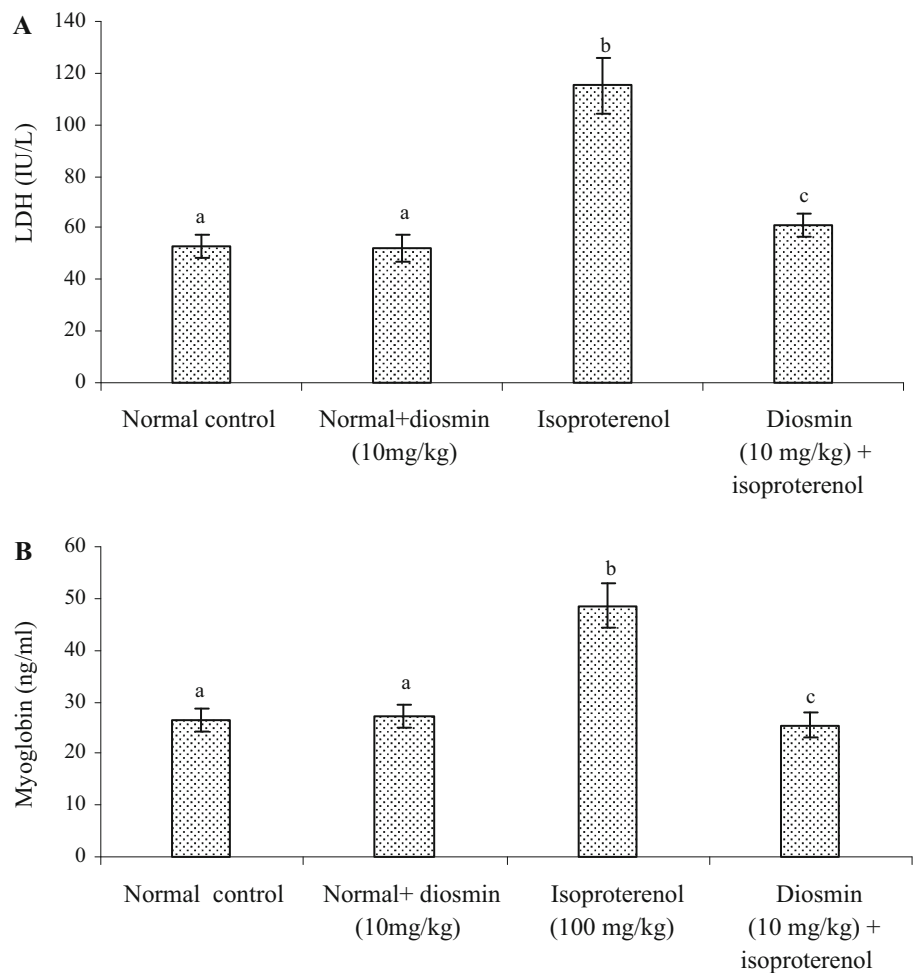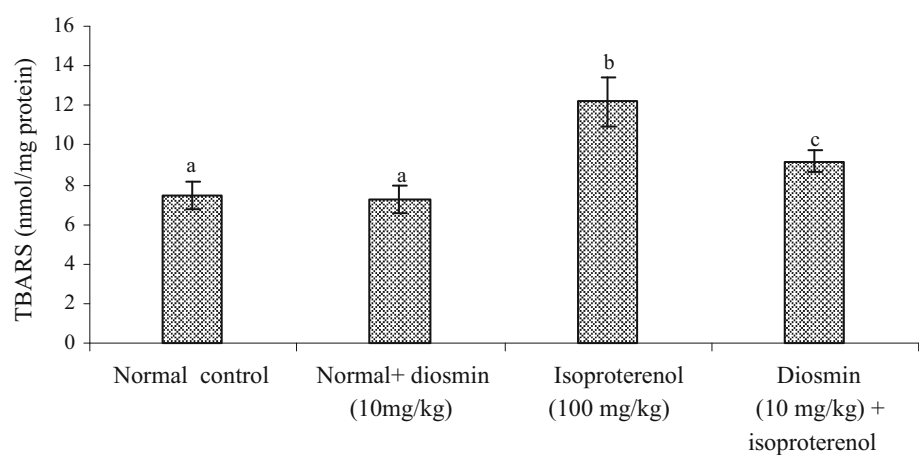

Fig. 2 Level of heart mitochondrial TBARS. Each column is the mean ± standard deviation; $n = 6$; columns sharing a common letter *(a, a)* do not differ significantly with each other; columns not sharing a common letter *(a–c)* differ significantly with each other ($P < 0.05$, Duncan's multiple range test)

the heart mitochondria compared with normal control rats. Oral pretreatment with diosmin significantly ($P < 0.05$) lowered the level of TBARS in the heart mitochondria of isoproterenol-induced myocardial infarcted rats compared to isoproterenol-alone-induced myocardial infarcted rats (Fig. 2).

Effect of Diosmin on the Level/Activity of SOD, GSH, GPx, and GST in the Heart Mitochondria

A significant ($P < 0.05$) decrease in the activity/level of SOD, GSH, GPx (Fig. 3a), and GST (Fig. 3b) was noted in the heart mitochondria of isoproterenol-induced myocardial infarcted rats compared with normal control rats. Pretreatment with diosmin significantly ($P < 0.05$) improved the above-mentioned antioxidant system in the heart mitochondria of isoproterenol-induced myocardial infarcted rats compared to isoproterenol-alone-induced myocardial infarcted rats.

Effect of Diosmin on the Activities of Heart Mitochondrial TCA Cycle Enzymes

The activities of TCA cycle enzymes such as ICDH, MDH (Fig. 4a), SDH, and α-KDH (Fig. 4b) were significantly ($P < 0.05$) decreased in the heart mitochondria of isoproterenol-induced myocardial infarcted rats compared to normal control rats. Pretreatment with diosmin enhanced significantly ($P < 0.05$) the above-mentioned TCA cycle enzymes in isoproterenol-induced myocardial infarcted rats compared with isoproterenol-alone-induced myocardial infarcted rats.

Effect of Diosmin on the Level of Ca^{2+} in the Heart Mitochondria

The level of Ca^{2+} was increased significantly ($P < 0.05$) in the heart mitochondrial fraction of isoproterenol-induced myocardial infarcted rats compared with normal control rats. Diosmin pretreatment significantly ($P < 0.05$) decreased the level of Ca^{2+} in the heart mitochondrial fraction of isoproterenol-induced myocardial infarcted rats compared with isoproterenol-alone-induced myocardial infarcted rats (Fig. 5).
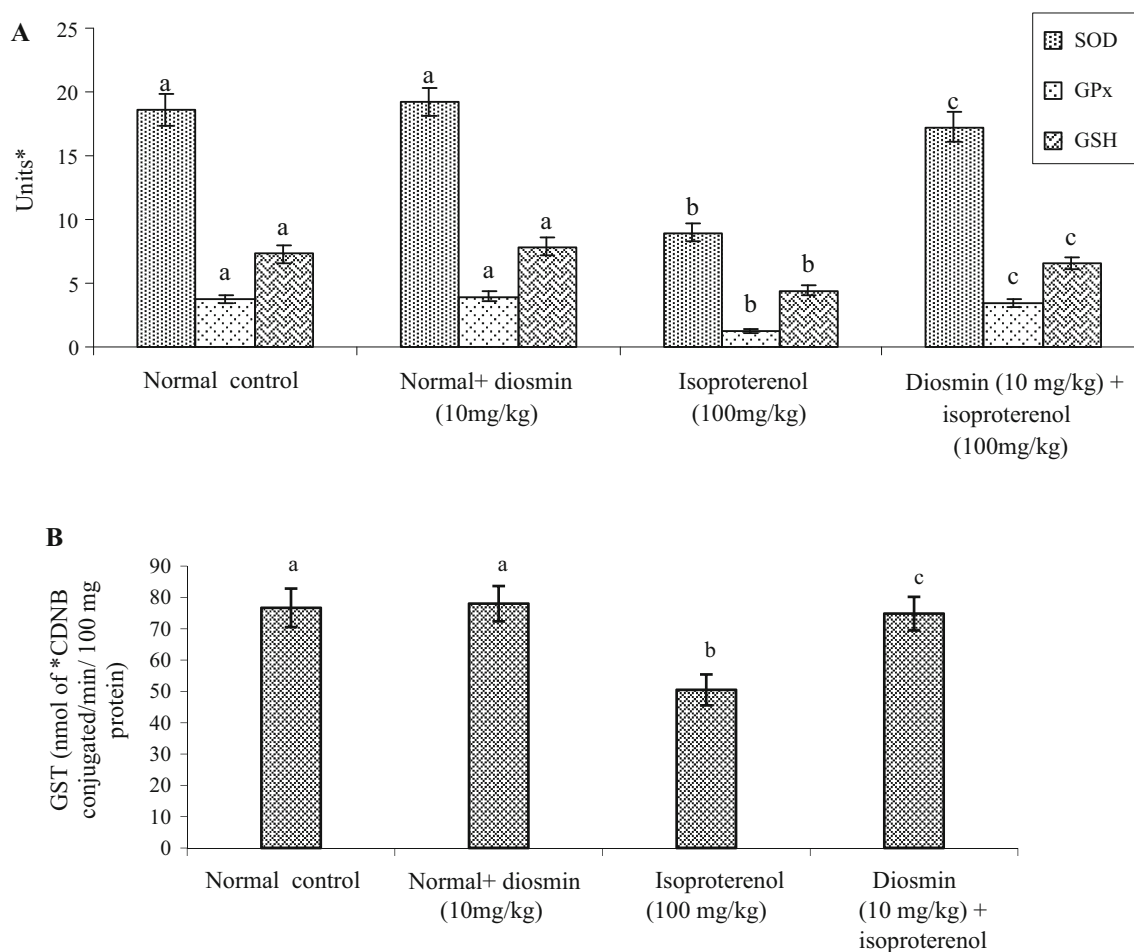

Fig. 3 a, b Activities/level of heart mitochondrial SOD, GSH, GPx and GST. Each column is the mean ± standard deviation; $n = 6$; columns sharing a common letter (a, a) do not differ significantly with each other; columns not sharing a common letter (a–c) differ significantly with each other ($P < 0.05$, Duncan's multiple range test); *Units/mg protein; One unit of SOD is defined as the enzyme concentration required inhibiting the optical density at 560 nm of chromogen production by 50% in one min; GSH: nmoles of GSH reduced/100 mg protein; GPx: nmoles of GSH oxidized/min/100 mg protein; CDND: 1-chloro-2,4-dinitrobenzene
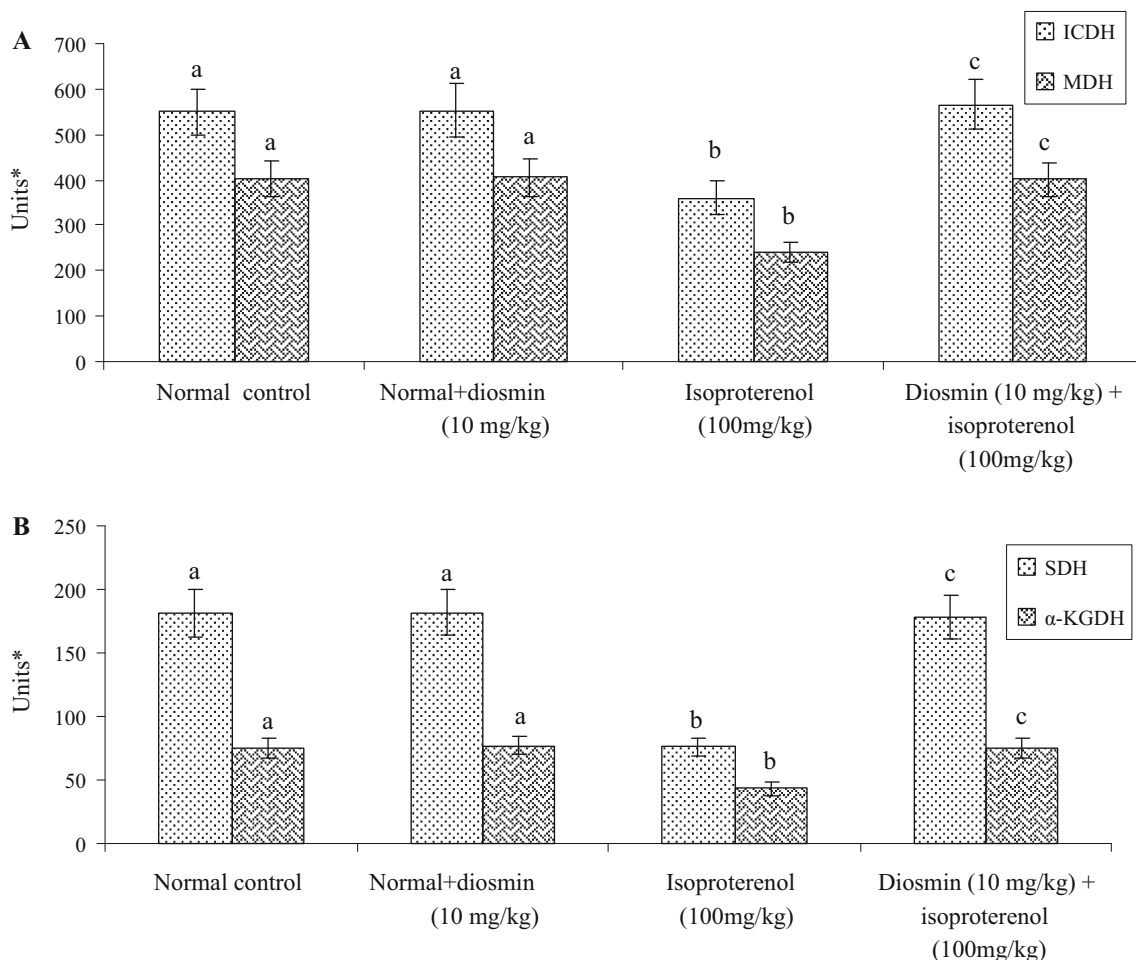
Fig. 4 a, b Activities of heart mitochondrial TCA cycle enzymes. Each column is the mean ± standard deviation; *n* = 6; columns sharing a common letter *(a, a)* do not differ significantly with each other; columns not sharing a common letter *(a–c)* differ significantly with each other (*P* < 0.05, Duncan's multiple range test). *Units: activity is expressed as nmoles of α-ketoglutarate formed/h/mg protein for ICDH; nmoles of nicotinamide adenine dinucleotide oxidized/min/mg protein for MDH; nmoles of ferrocyanide formed/h/mg protein for α-KDH; nmoles of succinate oxidized/min/mg protein for SDH

Fig. 5 Level of Ca^{2+} in heart mitochondria. Each column is the mean ± standard deviation; *n* = 6; columns sharing a common letter *(a, a)* do not differ significantly with each other; columns not sharing a common letter *(a–c)* differ significantly with each other (*P* < 0.05, Duncan's multiple range test)
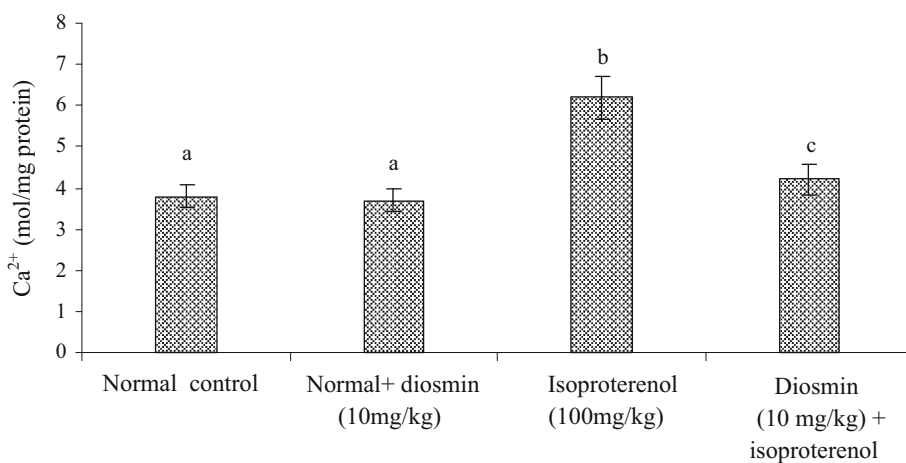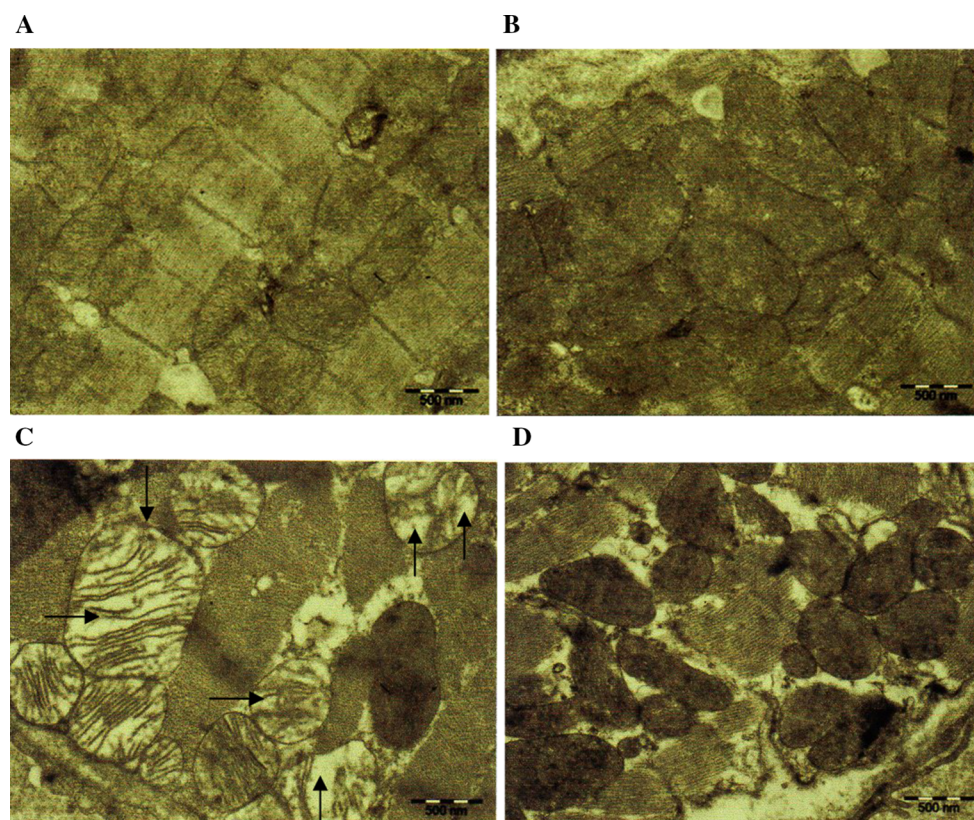
Fig. 6 a–d Transmission electron microscopic study on the structure of heart mitochondria. **a** Normal control rats heart (Group-I) showing normal architecture of mitochondria without any damage (20,000×); **b** diosmin-treated rats heart (Group-II) revealing normal architecture of mitochondria without any damage (20,000×); **c** isoproterenol-induced myocardial infarcted rats heart (Group-III) showing an increase in size, irregular shape, swelling ($\downarrow$) loss of cristae ($\rightarrow$) with vacuolation ($\uparrow$) in mitochondria (20,000×); **d** diosmin-pretreated isoproterenol-induced myocardial infarcted rats heart (Group-IV) showing near-normal architecture of mitochondria (20,000×)

Effect of Diosmin on the Structure of Heart Mitochondria (Transmission Electron Microscopic Study)

Transmission electron microscopic study revealed normal architecture of mitochondria in both normal control and diosmin-treated rats heart (Groups-I and II) (Fig. 6a, b). An increase in size, irregular shape, mitochondrial swelling ($\downarrow$), loss of cristae ($\rightarrow$), and vacuolation ($\uparrow$) were noted in isoproterenol-induced myocardial infarcted rats heart (Group-III) (Fig. 6c). Near-normal architecture of mitochondria was observed in diosmin-pretreated isoproterenol-induced myocardial infarcted rats heart (Group-IV) (Fig. 6d).

Effect of Diosmin on the Degree of Damage of Heart Mitochondria

The effects of diosmin on the degree of mitochondrial damage observed from transmission electron microscopic study (Fig. 6a–d) are shown in Table 1. The normal rats heart (Group-I) (Fig. 6a) and diosmin (10 mg/kg body

weight)-treated rats heart (Group-II) (Fig. 6b) revealed normal architecture of mitochondria without swelling (A), loss of cristae (A), and vacuolation (A). However, isoproterenol-induced myocardial infarcted rats heart (Group-III) showed mitochondrial swelling (++), loss of cristae (+++), and vacuolation (+++) (Fig. 6c). Diosmin (10 mg/kg body weight)-pretreated isoproterenol-induced myocardial infarcted rats heart (Group-IV) revealed absence of mitochondrial swelling (A), loss of cristae (A), and vacuolation (A) (Fig. 6d).

Effect of Diosmin on In Vitro Reducing Activity by FRAP Assay

Figure 7 reveals the results of in vitro FRAP of diosmin at various concentrations (10, 20, 30, 40 and 50 μM). From the findings, it is clear that the FRAP of diosmin increases with increasing concentrations. Thus, the in vitro study reveals the ferric reducing antioxidant power of diosmin.
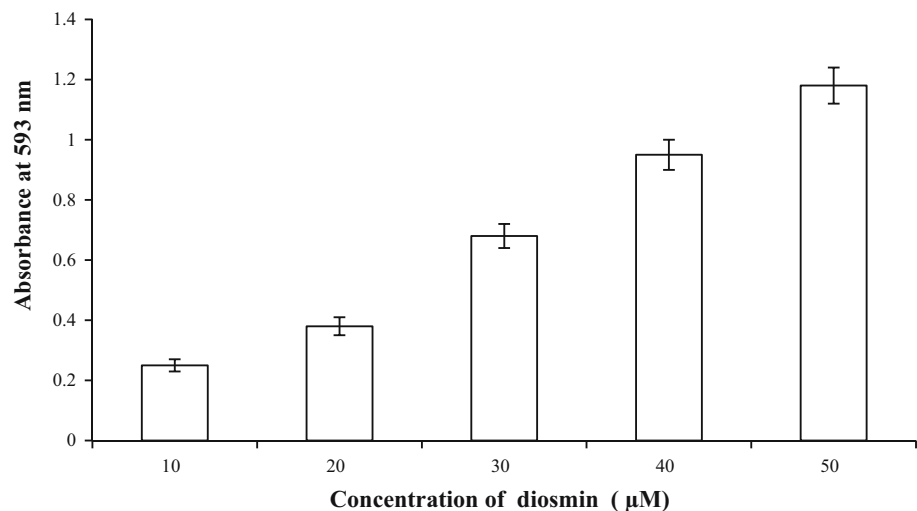
Diosmin (10 mg/kg body weight) administration to normal rats (Group-II) did not show any effect on the

Table 1 Effect of diosmin on the degree of transmission microscopic changes in the mitochondria of normal and experimental rats heart

Groups	Swelling	Loss of cristae	Vacuolation
Normal control	A	A	A
Normal + diosmin (10 mg/kg body weight)	A	A	A
Isoproterenol (100 mg/kg body weight)	++	+++	+++
Diosmin (10 mg/kg body weight) + isoproterenol (100 mg/kg body weight)	A	A	A

Photomicrographs (Fig. 6a–d) were used to evaluate the degree of damage in the mitochondria of heart; (A) no changes; (++) moderate changes; (+++) severe changes

Fig. 7 In vitro reducing (antioxidant) activity of diosmin by FRAP method. Each column is the average of triplicate experiments

Discussion

To the best of our knowledge, this is the first study performed on the preventive effects of diosmin on oxidative stress in the heart mitochondria of isoproterenol-induced myocardial infarcted rats. Isoproterenol-induced myocardial infarction in animals is a well-standardized and common model for assessing cardiac dysfunction and for studying the effectiveness of various cardioprotective agents/phytoconstituents in preclinical research. Reproducibility, technical simplicity and low morbidity and mortality are the notable characteristics of this model [24–27]. The strong inotropic and chronotropic actions of isoproterenol induce myocardial infarction in animals. In addition, there is no need of administering anesthesia and the possibility of entering foreign body in the heart of animals in this model as compared to the physical occlusion of the coronary artery model in animals [28]. Accumulated studies have revealed that the hemodynamic, electrophysiological, pathophysiological, biochemical, histopathological, and molecular changes occurring in the isoproterenol-

biochemical parameters and the structure of cardiac mitochondria studied, indicating this dose appears safe.

induced myocardial infarcted rat's heart are similar to that of human's myocardial infarcted heart [29–32].

In the first phase of our experiment, we observed pretreatment with diosmin (5 mg and 10 mg/kg body weight) daily for a period of 10 days showed antihyperlipidaemic effects in isoproterenol (100 mg/kg body weight)-induced myocardial infarcted rats. But the effect exerted by 10 mg/kg body weight of diosmin revealed the most significant effect as compared to the other dose 5 mg/kg body weight [8]. Hence, we have chosen 10 mg/kg body weight of diosmin for the present study.

Cardiac markers in blood are highly useful in detecting myocardial infarction. Lactate dehydrogenase and myoglobin are present in the myocardium, and these are the diagnostic markers of myocardial infarction. The elevation of these two cardiac markers in the blood is an indication of myocardial infarction. Hence, we have chosen these cardiac markers in this study. Myoglobin is one of the earliest markers released into the circulation after the onset of myocardial necrosis. It is released into the circulation as soon as 1 h after coronary occlusion because of its low molecular weight. The leakage of these diagnostic markers is due to the cardiotoxicity induced by isoproterenol. Pretreatment with diosmin (10 mg/kg body weight) daily for a period of 10 days prevented the cardiotoxicity induced by

isoproterenol and decreased the levels of above-mentioned diagnostic markers in isoproterenol-induced myocardial infarcted rats and protected the myocardium, by virtue of its cardioprotective effects.

The underlying mechanism of action of diosmin was studied in vivo. Oxidative stress is well documented in the pathogenesis of humans as well as isoproterenol-induced myocardial infarction. The alterations in cardiac function and ultrastructure in experimental rats is due to isoproterenol-induced oxidative stress. Pharmacotherapy to increase endogenous myocardial antioxidants is one of the best approaches in diseases associated with increased oxidative stress. Mitochondrial membrane contains huge quantity of polyunsaturated fatty acids in its phospholipids. Hence, mitochondria are very much prone to lipid peroxidation [33]. The observed increase in the oxidative stress marker such as TBARS in the heart mitochondria of isoproterenol-induced myocardial infarcted rats indicates oxidative stress, which is the main reason for myocardial mitochondria damage, and this may decrease mitochondrial membrane fluidity, increase negative surface charge distribution, and alter membrane ionic permeability including proton permeability, which uncouple oxidative phosphorylation in myocardial infarcted rats [34]. Thus, increased oxidative stress damages mitochondrial structure and causes mitochondrial dysfunction in isoproterenol-induced myocardial infarcted rats. Diosmin pretreatment lowered isoproterenol-induced cardiac mitochondrial lipid peroxidation. Thus, diosmin inhibited oxidative stress by scavenging overproduction of free radicals and protected heart mitochondria from damage.

Isoproterenol metabolism causes oxidative stress, thereby depleting endogenous antioxidant system. The increased superoxide radicals generated by isoproternol is the reason for the declined activity of SOD observed in the isoproterenol-induced cardiac mitochondria. GSH prevents lipid peroxidation mediated mitochondrial membrane damage [34]. Further, reduced mitochondrial GSH is a major mechanism for inducing mitochondrial dysfunction which is a clear indication of oxidative stress. The reduction in GSH level observed in isoproterenol-induced rats can cause membrane integrity loss, heart contractile dysfunction and cardiomyocyte toxicity, and finally myocardial necrosis. GSTs are phase-II detoxification enzymes. They detoxify xenobiotics and endogenous electrophiles [35]. The activities of GPx and GST were lessened due to the reduced availability of GSH because of oxidative stress in the isoproterenol-induced myocardial infarcted rat's heart mitochondria. Insufficient mitochondrial GPx activity promotes isoproterenol-induced damage that cause mitochondrial dysfunction [36]. Thus the observed lowered levels of these antioxidant systems are due to increased oxidative stress. Pretreatment with diosmin prevented

oxidative stress produced by isoproterenol and increased the activities/levels of the above-mentioned antioxidant system and ameliorated mitochondrial damage in isoproterenol-induced myocardial infarcted rats, by its antioxidant property.

ICDH, a vital enzyme in TCA cycle, is present in mitochondrial matrix. It catalyzes the reversible conversion of isocitrate to α-ketoglutarate and carbon dioxide. Another enzyme, MDH, is located in the outer membrane of mitochondria. It is involved in the conversion of oxaloacetate and malate. SDH is an enzyme found in the mitochondria which oxidizes succinate to fumarate in TCA cycle. In the electron transport chain, SDH converts ubiquinone to ubiquinol. In the mitochondria, α-KDH converts α-ketoglutarate into succinyl-CoA and nicotinamide adenine dinucleotide. The lowered activities of these TCA cycle enzymes in the cardiac mitochondria of myocardial infarcted rats are due to tissue hypoxia caused by isoproterenol [37]. Further, oxidative stress caused by isoproterenol is another reason for alteration in the activities of these enzymes. Pretreatment with diosmin improved the activities of these TCA cycle enzymes by inhibiting increased oxidative stress in isoproterenol-induced myocardial infarcted rats.

Positive inotropism is one of the main mechanisms of isoproterenol-induced myocardial infarction. Cardiac mitochondrial function is regulated by Ca^{2+}. The increased levels of cardiac mitochondrial Ca^{2+} is due to enhanced Ca^{2+} uptake by isoproterenol, and this can inhibit electron transport and oxidative phosphorylation and increase ROS generation [37]. Diosmin-pretreated isoproterenol-induced myocardial infarcted rats lowered Ca^{2+} overload, by its negative inotropic effects, and protected the cardiac mitochondria from oxidative stress.

A change in the mitochondrial morphology is a key indicator of cellular pathology. The mitochondrial damage is examined by a transmission electron microscope. Isoproterenol-induced rats heart showed damaged mitochondria with increased size, irregular shape, loss of cristae, swelling, and vacuolation. These changes might be due to increased oxidative stress, which is a clear evidence of mitochondrial damage. But diosmin pretreatment revealed near-normal mitochondrial architecture in myocardial infarcted rats. Thus, diosmin protected the cardiac mitochondrial structure and prevented mitochondrial damage by inhibiting oxidative stress, because of its antioxidant effects.

To know the mechanism of action, the reducing (antioxidant) activity of diosmin was assessed by FRAP in vitro assay. The in vitro study revealed the ferric reducing antioxidant activity of diosmin in a concentration-dependent manner. The reducing power of a compound reveals its potent antioxidant activity. Thus, the in vitro study confirmed the antioxidant property of diosmin.

Diosmin exhibits suitable pharmacokinetics. Rats were orally administered diosmin with 3 different doses as 225, 325 and 425 mg/kg body weight [38]. Then, the plasma concentration of diosmin was determined in the blood samples collected at different time intervals after oral administration by high performance liquid chromatography and the pharmacokinetics parameters of diosmin were determined. The typical equation of diosmin in rat's plasma was $Y = 3.05 \times 10^{-3}\,C + 1.55 \times 10^{-3}$, the calibration curves of diosmin was linear in the range from 0.5 to 100 μg/ml ($R = 0.9964$). The lowest concentration of diosmin in plasma was 0.2 g/ml. Diosmin was recovered more than 85%. After three doses oral administration of diosmin in rats, the mean plasma concentration–time curves were found to fit one compartment model, and the main pharmacokinetics parameters were obtained [38]. Thus, the above study clearly revealed that after oral administration of diosmin, it is present in the plasma, and more than 85% of diosmin is recovered [38]. In our study, diosmin present in the plasma after oral administration to rats, improved the endogenous antioxidant system and prevented oxidative stress, thereby reducing the intensity of heart mitochondrial damage in myocardial infarcted rats, by virtue of its antioxidant property.

Conclusions

In conclusion, diosmin attenuates cardiac mitochondrial oxidative stress in isoproterenol-induced myocardial infarcted rats and prevents mitochondrial damage, by virtue of its antioxidant and negative inotropic properties. This can also be evidenced from transmission electron microscopic study. These results are rational to understand the protective effects of diosmin on cardiac mitochondria against myocardial infarction, in which oxidative stress and inotropy is known to contribute to the pathogenesis. According to our findings, a 70-kg person requires 700 mg of diosmin/day. This dose is not very high. Hence, the dose diosmin 10 mg/kg body weight is physiologically relevant. However, clinical trials are needed to find out the exact dose of diosmin.

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