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α-Bisabolol abrogates isoproterenol-induced myocardial infarction by inhibiting mitochondrial dysfunction and intrinsic pathway of apoptosis in rats

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Abstract

Mitochondrial dysfunction plays crucial role in the pathologenesis of myocardial infarction (MI). The present study evaluated the protective effect of α-bisabolol against isoproterenol (ISO)-induced mitochondrial dysfunction and apoptosis in rats. Male albino Wistar rats were pre- and co-treated with intraperitoneal injection of α-bisabolol (25 mg/kg body weight) daily for 10 days. To induce experimental MI, ISO (85 mg/kg body weight) was injected subcutaneously to the rats at an interval of 24 h for 2 days (9th and 10th day). ISO-induced MI was indicated by the decreased activities of heart creatine kinase and lactate dehydrogenase in rats. ISO administration also enhanced the concentrations of heart mitochondrial lipid peroxidation products and decreased the activities/concentrations of mitochondrial antioxidants, Kreb's cycle dehydrogenases and mitochondrial electron transport chain complexes I, II + III and IV in rats. Furthermore, ISO triggers calcium overload and ATP depletion in the rat's heart mitochondria followed by the mitochondrial cytochrome-C release and the activation of intrinsic pathway of apoptosis by upregulating the myocardial pro-apoptotic Bax, P^{53}, APAF-1, active caspase-3, active caspase-9 and down regulating the expressions of anti-apoptotic Bcl-2. α-Bisabolol pre and co-treatment showed considerable protective effects on all the biochemical and molecular parameters studied. Transmission electron microscopic study and mitochondrial swelling assay confirmed our biochemical and molecular findings. The in vitro study on hydroxyl radical also revealed the potent free radical scavenging activity of α-bisabolol. Thus, α-bisabolol attenuates mitochondrial dysfunction and intrinsic pathway of apoptosis in ISO-induced myocardial infarcted rats.

Abbreviations

ISO	Isoproterenol
MI	Myocardial infarction
ATP	Adenosine triphosphate
Bax	B-cell lymphoma-2 associated-x
APAF-1	Apoptotic protease activating factor 1
Bcl-2	B-cell lymphoma-2
ETC	Electron transport chain
ROS	Reactive oxygen species
TCA	Tricarboxylic acid
Ca^{2+}	Calcium
CK	Creatine kinase
LDH	Lactate dehydrogenase
TBARS	Thiobarbituric acid reactive substances
LOOH	Lipid hydroperoxide
SOD	Superoxide dismutase
GSH	Reduced glutathione
ICDH	Isocitrate dehydrogenase
MDH	Malate dehydrogenase
SDH	Succinate dehydrogenase
α-KGDH	α-Ketoglutarate dehydrogenase
TEM	Transmission electron microscopy
OH$^{\bullet}$	Hydroxyl radical
DMRT	Duncan's multiple range test
SPSS	Statistical Package for the Social Science
SD	Standard deviation
NAD	Nicotinamide adenine dinucleotide

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NADH   Reduced nicotinamide adenine dinucleotide
NADP   Nicotinamide adenine dinucleotide phosphate

Introduction

Ischemic heart diseases manifested mainly as acute myocardial infarction (MI) is prevailing worldwide including developed as well as developing nations and often presents a socioeconomic burden with increasing disability. MI is defined as damage of the heart muscles emerging from continuous acute myocardial ischemia. The death of heart tissues in MI involves both necrosis and apoptosis with distinctive morphological changes and alterations in the cardiac function [1–3]. To mimic the human MI, there are many experimental animal models with a popular one wherein the subcutaneous administration of isoproterenol (ISO) at larger doses induces myocardial injury in laboratory animals similar to that seen in acute MI in humans. ISO is a synthetic catecholamine and β-adrenergic agonist and provides a rapid, simple and non-invasive method for the evaluation of numerous agents for their cardioprotective effects [2, 3]. ISO triggers the β-adrenergic receptor activation which possesses a pivotal role in modified cardiac contraction and energy metabolism during oxidative stress conditions and it finally regulates the progression of myocardial remodeling and failure [4, 5]. At higher doses, ISO induces severe oxidative stress in the myocardium of rats characterized by gross and microscopic infarct like lesions [6–9].

Among many mechanisms of oxidative stress, impaired mitochondrial function is one of the most significant sources of reactive oxygen species (ROS) production in the heart and it is usually associated with cardiac dysfunction that further leads MI and heart failure [10]. Synthesis of adenosine triphosphate (ATP) and electron transport chain (ETC) required for cardiac contraction and relaxation are located in the heart mitochondria. The generation of ROS in the failing myocardium triggers mitochondrial DNA damage and consequent cellular injury leading to functional decline. Thus, mitochondrion is serving as a target and source for ROS mediated myocardial injury [11]. Excessive ROS production stimulates cellular dysfunction and causes damage to the electron transport complexes and respiration impairment. The role of energy metabolism in the mitochondria, cell signaling and programmed cell death are the main reasons for focusing the mitochondria as a suitable therapeutic approach to protect the myocardium against ISO-induced MI [12]. During ischemic conditions, the impairment of mitochondrial respiratory chain provides a new therapeutic approach for therapeutic targeting.

Growing evidences have revealed that apoptosis, a highly organized form of cell death participates in ischemic heart diseases including myocardial damage, heart failure and hypertrophy [13]. Oxidative stress has been shown to activate multiple cell signaling pathways including apoptosis [14]. The events of mitochondrial apoptotic pathway are controlled via the proteins of Bcl-2 family which govern the permeability of mitochondrial membrane [15]. The P^{53} possess a vital role in regulation of the Bcl-2 family proteins and those actions are leading to the activation of caspases and finally cell death [16]. Since apoptosis plays an important role in cell death of cardiomyocytes in MI, thus inhibition of apoptosis could be an important therapeutic target for the treatment of ischemic heart diseases.

α-Bisabolol is a sesquiterpene alcohol isolated from the essential oil of variety of plants, shrubs and trees. It is abundantly found in the essential oil of *Matricaria chamomilla* (*Asteraceae*), popularly known as chamomile, which contains up to 50% of α-bisabolol [17]. Generally, α-bisabolol is extracted by hydrodistillation of the respective essential oils from sage (*Salvia runcinata*), German chamomile (*M. chamomilla*), *Vanillosmopsis* sp. and *M. crassifolium* [18]. It is a small molecule with favorable physicochemical and pharmaceutical properties in terms of drug discovery and development. It possesses a wide range of pharmacological properties such as anti-inflammatory [19], antibiotic [20], gastroprotective [21], antioxidant [22] and antimutagenic properties [23]. The United States Food and Drug Administration (US FDA) has classified α-bisabolol as 'generally regarded as safe' (GRAS) due to its low toxicity that encourage its use as an active ingredient in commercial products [17]. A recent study from Corpas-Lopez et al. [24] has revealed that repeated administration of α-bisabolol did not show any signs of toxicity in hamsters. In spite of the fact that α-bisabolol has been recognized for its multipharmacological properties to treat other human diseases, its conceivable cardioprotective capability has not yet been known. Therefore, our study mainly focuses on evaluation of the α-bisabolol against ISO-induced MI in rats.

Since, mitochondrial dysfunction and apoptosis both are common accompaniment and intimately involved in the pathogenesis of MI, we carried out this study in ISO-induced MI in rats to investigate the effect of α-bisabolol on mitochondrial dysfunction by measuring the enzyme activities and levels of cardiac diagnostic markers, heart mitochondrial lipid peroxidation, antioxidants, tricarboxylic acid (TCA) cycle enzymes and inner mitochondrial respiratory chain complexes as well as calcium (Ca^{2+}) homoeostasis and intrinsic apoptotic pathway. Furthermore, the effect of α-bisabolol on the structure of heart mitochondria was revealed by transmission electron microscopic study and the free radical scavenging activity of α-bisabolol was assessed in vitro.

Materials and methods

Experimental animals

The experiment was performed with male albino Wistar rats weighing 160–180 g acquired from the experimental animal research facility of the College of Medicine & Health Sciences (CMHS), United Arab Emirates University, Al Ain, United Arab Emirates. A maximum of four rats were housed per cage in polypropylene cages ($47 \times 34 \times 20$ cm) lined with husk (replaced every 24 h). The animals were housed under standard laboratory conditions of a 12 h light/dark cycle at around 22°C. Rats were fed on standard rodent chow diet (National Feed and Flour Production and Marketing Company LLC., Abu Dhabi, UAE) and water ad libitum. The experimental protocol (A31/14) for animal experimentation was approved by the Animal Ethics Committee of United Arab Emirates University.

Drugs and chemicals

α-Bisabolol, ISO hydrochloride, nitroblue tetrazolium, phenazine methosulphate, 1-chloro-2,4-dinitro benzene, potassium dichromate, 2,4-dinitro phenyl hydrazine, xylenol orange, glutathione, α-ketoglutarate, thiamine pyrophosphate, sodium succinate, bovine serum albumin, oxaloacetate, cytochrome-C and decylubiquinone were purchased from Sigma Chemical Company, St. Louis, MO, USA. All other chemicals used were of analytical grade.

Induction of experimental MI

ISO (85 mg/kg body weight) dissolved in saline was subcutaneously injected into rats at an interval of 24 h for 2 days [25–28]. ISO-induced MI was confirmed by decreased activities of heart creatine kinase (CK) and lactate dehydrogenase (LDH) in rats.

Experimental design

The animals were divided into four experimental groups, each containing 15 rats. Group I: normal control rats; Group II: rats were intraperitoneally treated with α-bisabolol (25 mg/kg body weight) daily for a period of 10 days; Group III: rats were subcutaneously injected with ISO (85 mg/kg body weight) at an interval of 24 h for 2 days (on 9th and 10th day); Group IV: rats were pre- and co-treated intraperitoneally with α-bisabolol (25 mg/kg body weight) daily for a period of 10 days and were subcutaneously injected with ISO at an interval of 24 h for 2 days (9th and 10th day). The dose and the mode of administration of α-bisabolol was

selected based on our previous pilot study wherein we have screened many doses of α-bisabolol with different treatment protocol using different routes of administration [29]. Twelve hours after the second dose of ISO injection (i.e. on 11th day), the rats were anesthetized by pentobarbital sodium (60 mg/kg body weight) and then sacrificed by cervical decapitation. For serum, blood was collected in dry tubes without anticoagulant. The heart was excised immediately and rinsed in ice chilled saline. The heart tissue with determined weight were homogenized in Tris–HCl buffer (0.1 M; pH 7.4) and used for biochemical estimations. For western blotting, the heart tissues were homogenized in RIPA buffer. The heart tissues were also used for TEM and mitochondrial swelling studies.

Biochemical estimations

Assay of cardiac marker enzymes

The activity of CK and LDH enzymes were assayed using Vet Test 8008 Chemistry Analyzer, UK.

Isolation of heart mitochondria

The mitochondrial fraction of the heart tissue was isolated by the mitochondrial isolation kit purchased from Abcam, MA, USA.

Estimation of mitochondrial lipid peroxidation products

The concentration of thiobarbituric acid reactive substances (TBARS) in the heart mitochondrial fraction was estimated by the method of Fraga et al. [30]. 2 mL of thiobarbituric acid–trichloro aectic acid–hydrochloric acid reagent was mixed with 1 mL of mitochondrial fraction and kept in a boiling water bath for 15 min. After cooling, the tubes were kept for brief centrifugation and the fluorescence on the butanol layer was measured at 535 nm against the reagent blank.

The levels of lipid hydroperoxide (LOOH) in the heart mitochondrial fraction were estimated by the method of Jiang et al. [31]. For this assay, 0.2 mL of the heart mitochondrial fraction was mixed with 1.8 mL of Fox reagent and incubated at room temperature for 30 min. The developed chromophore was read at 560 nm.

Estimation of mitochondrial antioxidant enzymes

Superoxide dismutase (SOD) activity in the heart mitochondrial fraction was assayed by the method of Kakkar et al. [32]. For this assay, 0.5 mL of heart mitochondrial fraction was diluted to 1.0 mL with distilled water. Then, 1.5 mL of chloroform and 2.5 mL of ethanol (all chilled) were mixed

and centrifuged. The activity of enzyme in the supernatant was measured. To the assay mixture contained 1.2 mL of sodium pyrophosphate buffer, 0.3 mL of nitroblue tetrazolium and 0.1 mL of phenazine methosulfate and 0.2 mL of NADH, appropriately diluted enzyme preparation was added and make up to total volume of 3.0 mL with distilled water. Following incubation for 90 s at 30 °C, 1.0 mL of glacial acetic acid was added to arrest the reaction. The mixture was vigorously stirred and shaken with 4.0 mL of *n*-butanol. The chromogen intensity was measured at 560 nm against butanol blank.

The activity of catalase in the heart mitochondrial fraction was assayed by the method of Sinha [33]. Briefly described, 0.1 mL of heart mitochondrial fraction, 0.9 mL of phosphate buffer, 0.4 mL of hydrogen peroxide was added. After incubation for 60 s at room temperature, 2.0 mL of dichromate-acetic acid mixture was added. The tubes were kept in a boiling water bath for 10 min and the colour developed was read at 620 nm.

The levels of glutathione (GSH) in the heart mitochondrial fraction were performed by the method of Ellman [34]. For this assay, 0.5 mL of mitochondrial fraction was allowed to precipitate with 2.0 mL of 5% TCA. After centrifugation, 1.0 mL of the supernatant was taken and added 0.5 mL of Ellman's reagent and 3.0 mL of phosphate buffer. The yellow colour developed was read at 412 nm.

Assay of Kreb's cycle dehydrogenases

The activity of isocitrate dehydrogenase (ICDH) in the heart mitochondrial fraction was assayed in the heart mitochondria by the method of King [35]. To the assay mixture (0.4 mL of Tris–HCl buffer, 0.2 mL of manganese chloride, 0.2 mL of substrate and 0.2 mL of NADP), 0.2 mL of mitochondrial fraction was added and mixed well. 0.2 mL of saline was used instead of NADP for control. The tubes were incubated in room temperature for 1 h. After incubation period, 1.0 mL of coloring reagent (0.001 M; 2,4-dinitro phenyl hydrazine) and 0.5 mL of ethylene diamine tetraacetic acid were added and mixed well. The tubes were incubated at 37 °C for 20 min and 10 mL of sodium hydroxide (0.4 N) was added. The colour intensity was measured at 420 nm.

The activity of malate dehydrogenase (MDH) in the heart mitochondrial fraction was assayed by the method of Mehler et al. [36]. To the assay mixture (0.75 mL of phosphate buffer, 0.75 mL of oxaloacetate and 0.15 mL of NADH), 0.2 mL of mitochondrial fraction was added and the reaction was done at 25 °C. The control tubes contained all reagents except NADH and the change in absorbance was measured for 2 min at 340 nm at an interval of 15 s.

The succinate dehydrogenase (SDH) activity in the heart mitochondrial fraction was assayed by the method of Slater and Borner [37]. The reaction mixture contains 1.0 mL of phosphate buffer, 0.1 mL of sodium cyanide, 0.1 mL of ethylene diamine tetra-acetic acid, 0.3 mL of sodium succinate, 0.2 mL of potassium ferricyanide, 0.1 mL of bovine serum albumin was made up to 2.8 mL with distilled water. Then, 0.2 mL of mitochondrial fraction was added to initiate the reaction. The change in optical density was measured at 420 nm for 5 min at 15 s intervals.

The activity of α-ketoglutarate dehydrogenase (α-KGDH) in the heart mitochondrial fraction was assayed by the method of Reed and Mukherjee [38]. The incubation mixture contains 0.1 mL of thiamine pyrophosphate, 0.1 mL potassium α-ketoglutarate, 0.1 mL of phosphate buffer, 0.1 mL of magnesium sulfate and 0.1 mL of potassium ferricyanide was made up to 1.4 mL with distilled water followed by the addition of mitochondrial fraction. The control tube contains all the reagents except mitochondrial fraction. The mixture was allowed for incubation at 30 °C for 30 min. after incubation period, 1.0 mL of 10% TCA was added to terminate the reaction. After the addition of 10% TCA, the reaction mixture was mixed well and centrifuged. To 1.0 mL of supernatant, 1.5 mL of distilled water, 1.0 mL of 10% TCA and 1.0 mL of 4% duponol and 0.5 mL of ferric ammonium sulfate-duponol reagent were added. The tubes were kept for 30 min at room temperature and the absorbance was measured at 540 nm.

Assay of mitochondrial respiratory chain complexes

The activities of inner mitochondrial ETC complexs-I, II + III were determined by the method of Kramer et al. [39].

The complex-I (rotenone sensitive NAD-coenzyme Q reductase) activity was determined with two different 2-point rate assays (4 min) by measuring the total and rotenone-insensitive complex I activities. To 19 µL of mitochondrial fraction, 350 µL of substrate solution (pH-7.2) (27 mmol of potassium phosphate buffer, 2.66 g/L bovine serum albumin, 5 mmol/L magnesium chloride, 0.213 mmol/L NADH, 2 mmol/L potassium cyanide, 0.1065 mmol/L decylubiquinone, 0.002 g/L antimycin A) was added and the decreased absorbance was monitored. The activity of rotenone-insensitive complex-I was measured by adding 39.9 µmol/L rotenone with 350 µL of substrate solution and the decreased absorbance was monitored. The difference between the activity of total and rotenone insensitive complex was used to calculate complex-I enzyme activity.

The activity of complex-II + III: Succinate cytochrome-C reductase was measured with 2-point rate assay (5 min). Mitochondrial fraction (50 µL) was added to 300 µL

of substrate solution (pH-7.8) (37.5 mmol/L potassium phosphate, 25 mmol/L succinic acid, 1.87 mmol/L potassium cyanide, 0.005 g/L rotenone and 0.5 mmol/L ethylene diamine tetraacetic acid). Then 37 μL of 0.5 mmol/L cytochrome-C was added after 1.5 min. The increased absorbance was read at 546 nm to calculate the complex II + III enzyme activity.

The activity of cytochrome-C-oxidase in the heart mitochondrial fraction was assayed by the method of Pearl et al. [40]. The assay was done by adding the reaction mixture containing 1.0 mL of phosphate buffer, 0.1 mL of cytochrome-C (0.01%), 0.2 mL of N-phenyl-p-phenylene diamine (0.2%) and 0.5 mL of distilled water. Then, 0.2 mL of mitochondrial fraction was added and the absorbance was recorded for 5 min (interval of 15 s each) at 550 nm. The tube contains all the reagents except cytochrome-C was used as control.

Estimation of Ca^{2+} and ATP

The heart mitochondrial Ca^{2+} levels were measured by the calcium colorimetric assay kit obtained from Sigma Chemical Company, St. Louis, MO, USA. The heart mitochondrial ATP concentration was measured by the method of Williams and Coorkey [41]. To the incubation mixture contains 2 mL of phosphate buffer (pH 7.4), 5 mM ethylene diamine tetraacetic acid, 10 mM magnesium chloride, 10 μL of adenosine diphosphate, 5 μL of glucose-6-phosphate dehydrogenase; 0.2 mL of mitochondrial fraction was added. Mixed thoroughly and the fluorescence was recorded. Then, 10 μL of ATP and 5 μL of hexokinase were added and the increased absorbance was recorded at 340 nm.

Mitochondrial swelling assay

The mitochondrial permeability transition pore activation in isolated cardiac mitochondria was measured by Ca^{2+}-induced swelling by the method of Maloyan et al. [42]. Opening of the pore results in swelling of the mitochondria and the decrease in absorbance was measured at 540 nm. Mitochondrial fraction was resuspended in the swelling buffer contains ten Tris–HCl (pH 7.4), 120 mmol/L potassium chloride and five potassium dihydrogen phosphate to a final protein concentration of 250 μg/mL. The pore opening was induced by 250 μmol/L of calcium chloride and measured at 540 nm.

Western blot analysis

Western blotting analysis was performed to analyze the expression pattern of pro-apoptotic B-cell lymphoma-2 associated-x (Bax), anti-apoptotic B-cell lymphoma-2 (Bcl-2), P[53], apoptotic protease activating factor 1 (APAF-1), active caspase-3, active caspase-9, cytochrome-C and β-actin. The heart tissue samples were homogenized in an ice-cold RIPA buffer and the homogenate was centrifuged at 2800×g/min for 30 min at 4 °C to remove debris. The protein contents in the sample were estimated using the Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). The protein samples were loaded and separated by SDS-polyacrylamide (12%). The membranes were incubated with the blocking buffer containing 5% bovine serum albumin for 1 h to reduce non-specific binding sites. The proteins were transferred onto PVDF membrane and incubated with specific primary antibodies; 1:500 dilution for Bax (Santa Cruz Biotechnology, Dallas, TX, USA), Bcl2, P[53], APAF-1, active caspase-3 (1:1000; Abcam, MA, USA), active caspase-9 (1:1000; Cell signalling technology, Danvers, MA, USA), voltage dependent anion channel (VDAC) (1:5000; Sigma St. Louis, USA) and β-actin (1:5000; MERCK Millipore, USA) with gentle shaking overnight at 4 °C. Then, membranes were incubated with their corresponding secondary antibodies (anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase) for 1 h at room temperature and the protein bands were visualized using an enhanced chemiluminescence pico kit (Thermo Fisher Scientific, Rockford, IL, USA). The intensity of bands was quantified using Image J software public domain Java image processing software, Wayne Rasband, NIH, Bethesda, MD, USA, which of control was set to 1.

Estimation of protein in the heart mitochondrial fraction

Protein content in the mitochondrial fraction was estimated using Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA).

Transmission electron microscopic study

The ultrastructure of the heart mitochondrial specimen was examined by transmission electron microscopy (TEM) (Fei, Teenai BioTwin G2, Holland) according to the method of Lang [43]. Small pieces of heart tissues were taken and rinsed in 0.1 M phosphate buffer (pH 7.2). Approximately, 1-mm heart pieces were trimmed and immediately fixed into 3% ice-cold glutaraldehyde in 0.1 M phosphate buffer (pH

7.2) and kept at 4 °C for 12 h. Then, tissue processing for TEM study 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.

The in vitro study

The free radical scavenging effect of α-bisabolol in vitro on hydroxyl radical (OH•) was determined by the method of Halliwell et al. [44]. Varying volumes of α-bisabolol (10, 20, 30, 40 and 50 µM) was mixed with 0.1 mL of 100 mM potassium phosphate buffer, 0.2 mL of 500 mM ferric chloride, 0.1 mL of 10 mM hydrogen peroxide, 0.1 mL of 1 mM ethylene diamine tetraacetic acid, 0.1 mL of 1 mM ascorbic acid, and 0.2 mL of 2-deoxyribose. The contents were mixed well and incubated for 60 min at room temperature. Then, 1 mL of 1% thiobarbituric acid in 0.05 N sodium hydroxide and 1 mL of 28% trichloroacetic acid was added and kept in a boiling water bath for 30 min. The absorbance was measured at 532 nm with distilled water as blank. Decreased absorbance of the mixture indicates increased OH• scavenging activity.

$$\% \text{ Scavenging of OH}^\bullet = \frac{\text{control O.D.} - \text{test O.D.}}{\text{control O.D.}} \times 100$$

Statistical analysis

Statistical analysis was performed by one-way analysis of variance followed by Duncans multiple range test (DMRT) using Statistical Package for the Social Science (SPSS) software package version 12.00. Results were expressed as mean ± standard deviation (SD) for eight rats in each group. The criterion of statistical significance was set on P values < 0.05.

Results

### α-Bisabolol protects the myocardium by preventing the leakage of CK and LDH

ISO-induced MI was confirmed by decreased activities of heart CK and LDH in rats compared to normal control rats. Pre and co-treatment with α-bisabolol daily for a period of 10 days significantly ($P < 0.05$) increased the activities of heart CK and LDH in ISO-induced rats compared to ISO control rats (Fig. 1a, b).
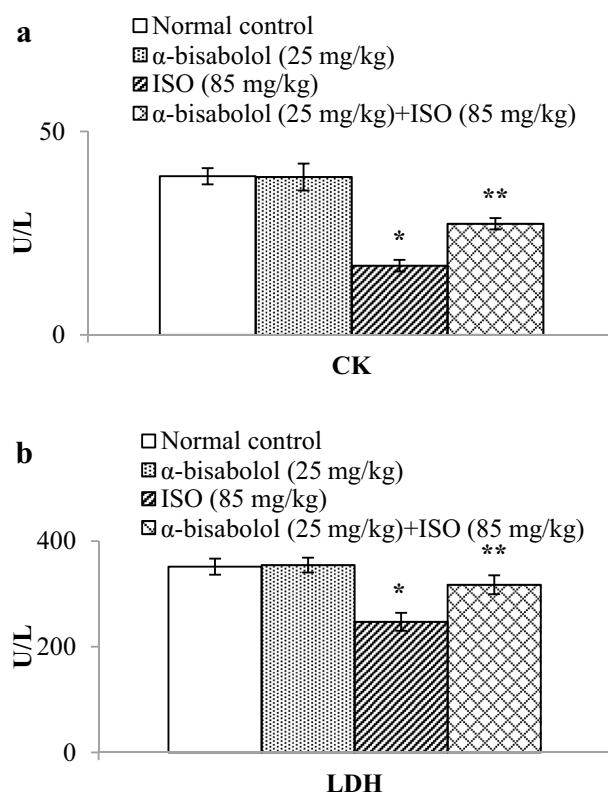
Fig. 1 Activities of CK and LDH in the heart. **a** Activity of CK in the heart. **b** Activity of LDH in the heart. Each column is mean ± SD for eight rats in each group; *$P < 0.05$ as compared to normal control (Group-I), **$P < 0.05$ as compared to ISO control (Group-III) (DMRT)

α-Bisabolol attenuates lipid peroxidation and maintained the antioxidant status

ISO-induced rats revealed significant ($P < 0.05$) increased levels of mitochondrial TBARS and LOOH with significant ($P < 0.05$) decreased activities/levels of SOD, catalase and GSH in the heart mitochondria compared to normal control rats. Pre and co-treatment with α-bisabolol revealed significant ($P < 0.05$) decreased levels of mitochondrial TBARS and LOOH and significant ($P < 0.05$) increased activities/levels of SOD, catalase and GSH in the heart mitochondria of ISO-induced rats compared to ISO control rats (Fig. 2a, b).
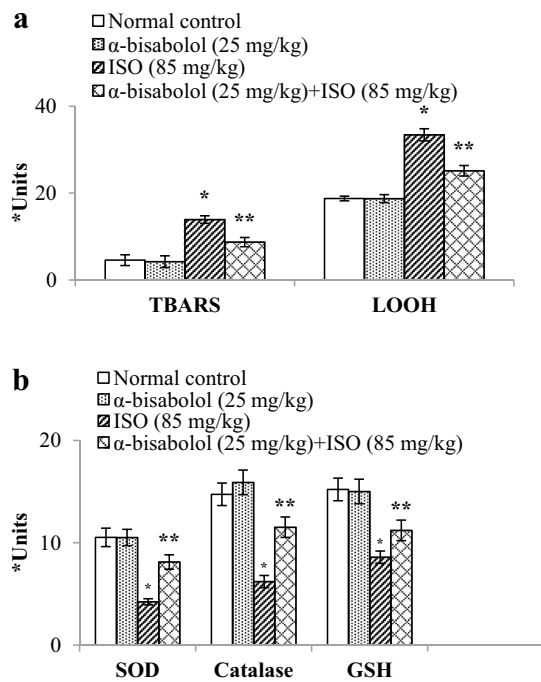
Fig. 2 Measurement of oxidative stress in heart mitochondria. **a** Levels of TBARS and LOOH in the heart mitochondria. **b** Activities/levels of SOD, catalase and GSH in the heart mitochondria. Each column is mean ± SD for eight rats in each group; *$P < 0.05$ as compared to normal control (Group-I), **$P < 0.05$ as compared to ISO control (Group-III) (DMRT). *Units: nM/mg protein for TBARS; nM/100 mg protein for LOOH; units/100 mg protein for SOD; nM of H_2O_2 consumed/min/mg protein for catalase; nM /100 mg protein for GSH

α-Bisabolol defends the myocardium by maintaining the status of Krebs cycle dehydrogenases and inner mitochondrial ETC complexes

The activities of Kreb's cycle dehydrogenases such as ICDH, SDH, MDH, α-KGDH and inner mitochondrial ETC complexes I, II + III and IV in the heart mitochondria were significantly ($P < 0.05$) decreased in ISO-induced rats compared to normal control rats. Pre and co-treatment with α-bisabolol showed significant ($P < 0.05$) increased activities of these enzymes in ISO-induced rats compared to ISO control rats (Fig. 3a–d).

α-Bisabolol reduced Ca²⁺ overload, mitochondrial swelling and increased ATP production in the mitochondria

The levels of Ca^{2+} and the swelling of mitochondria were considerably increased and the levels of ATP were considerably ($P < 0.05$) decreased in the heart mitochondria of ISO-induced rats. α-Bisabolol pre and co-treatment revealed significant ($P < 0.05$) decreased levels of Ca^{2+} and mitochondrial swelling with significant ($P < 0.05$) increased levels of ATP in the heart mitochondria of ISO-induced rats compared to ISO control rats (Fig. 4a, b).

## α-Bisabolol abrogates intrinsic pathway of apoptosis by preventing the mitochondrial cytochrome-C release

ISO administration showed upregulated expressions of Bax, P[53], APAF-1, active caspase-3, active caspase-9 and downregulated expression of Bcl-2 in the myocardium. Furthermore, ISO triggered the release of cytochrome-C from mitochondria to the cytosol. α-Bisabolol pre and co-treatment downregulated the expressions of pro-apoptotic proteins, such as Bax, P[53], APAF-1, active caspase-3, active caspase-9, and downregulated the expression of anti-apoptotic Bcl-2 protein (Fig. 5a, b). Also, α-bisabolol prevents the release of cytochrome-C from mitochondria to the cytosol (Fig. 6a, b). The results have clearly revealed that α-bisabolol abrogates the mitochondrial pathway of apoptosis by efficiently modulating the pro-apoptotic and anti-apoptotic markers in ISO-induced MI in rats.

TEM study on heart mitochondria

The TEM image of α-bisabolol alone treated rats showed normal mitochondrial architecture with intact myofibrils (Fig. 7a). ISO-induced mitochondria showed swelling, loss of cristae, irregular size and shape, vacuolation and loss of myofibrils heart of rats (Fig. 7b). α-Bisabolol pre and co-treated ISO-induced rats showed near normal architecture of mitochondria with intact myofibrils (Fig. 7c).

α-Bisabolol showed potent OH˙ scavenging activity

Figure 8 shows the percentage in vitro radical scavenging effects of α-bisabolol on OH˙. α-Bisabolol scavenges OH˙ in vitro in a concentration-dependent manner. The percentage scavenging effects of α-bisabolol on OH˙ at various concentrations (10, 20, 30, 40 and 50 μM) were found to be 17.3, 29.2, 55.3, 71.2 and 79.96, respectively. Thus, α-bisabolol at the concentration of 50 μM showed highest
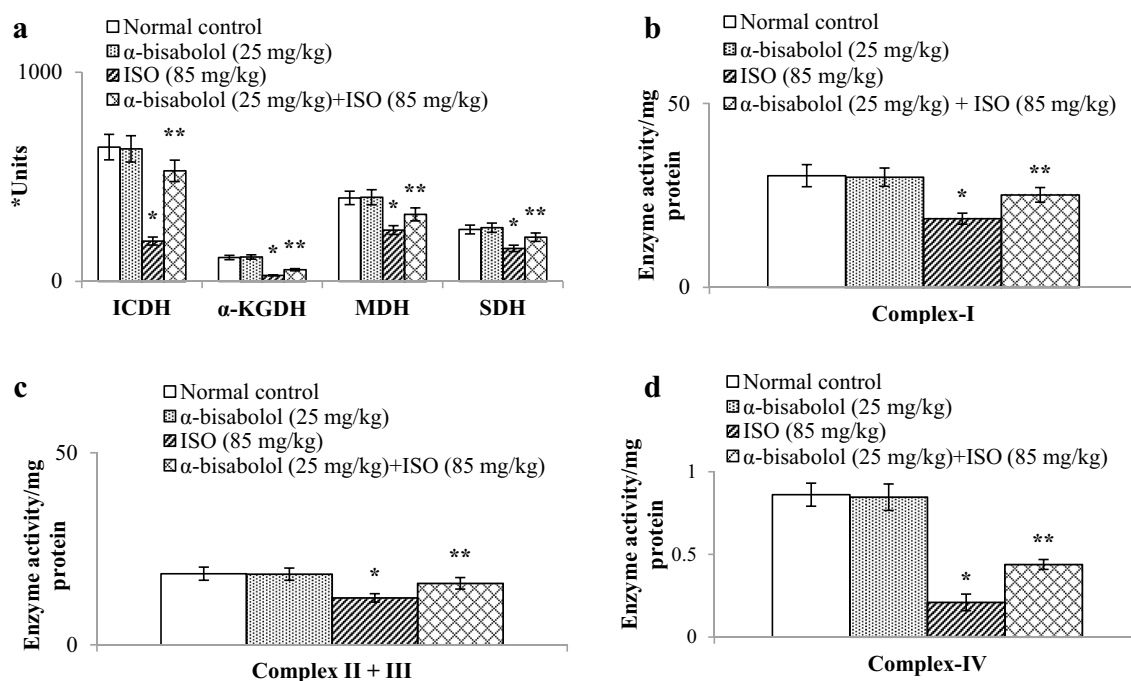
Fig. 3 Activities of TCA cycle and mitochondrial ETC enzymes. **a** Activities of TCA cycle enzymes in the heart mitochondria. *Units: nM of NADH oxidized/h/mg protein for ICDH; nM of ferro cyanide formed/h/mg protein for α-KGDH; nM of NADH oxidized/min/mg protein for MDH and nM of succinate oxidized/min/mg protein for SDH. **b** Activity of complex-I in the heart mitochondria. **c** Activity of complex-II + III in the heart mitochondria. **d** Activity of complex-IV in the heart mitochondria. Each column is mean ± SD for eight rats in each group; *$P < 0.05$ as compared to normal control (Group-I), **$P < 0.05$ as compared to ISO control (Group-III) (DMRT)

percentage scavenging effect of OH$^\bullet$ around 79.96%. Thus, α-bisabolol is a potent free radical quencher (Fig. 8).

Discussion

Subcutaneous injection of ISO-induced MI in rats make a more suitable experimental model for the evaluation of cardioprotective agents due to low mortality, high reproducibility and validity compared with other animal models [2, 3]. ISO by pharmacological stimulation induces MI in rats conveniently due to the smaller size of coronary arteries and activation of β-adrenergic G-protein coupled receptor after ISO-induced cardiac damage is responsible for the changes in the integrity and/or permeability of the plasma membrane results in the leakage of cardiac diagnostic marker enzymes [8]. It could be due to the sarcolemmal damage induced by β-receptor agonist and rendering it leaky [1]. During hypoxic conditions, myocardial cells are vulnerable for damage which results in the leakage cardiac diagnostic markers such as CK and LDH into the serum which reflects

the lowered CK and LDH activity in the myocardium [45]. α-Bisabolol appear to protects ISO challenged myocardium by preventing the leakage of myocardial CK and LDH into the circulation.

ISO-induced oxidative stress alters the function of myocardium and its ultrastructure in rats [46]. The generation of free radicals after myocardial injury plays a vital role in the progression of the damage [47]. The mitochondrial damage mediated by lipid peroxidation has been observed in ISO-induced MI in rats. Increased index markers of lipid peroxidation such as TBARS and LOOH may decrease the mitochondrial membrane fluidity and alters the proton permeability which uncouples the oxidative phosphorylation in the myocardium [46]. Thus, enhanced lipid peroxidation damages both the structure and function of mitochondria in ISO-induced rats. α-Bisabolol counters free radical mediated lipid peroxidation in ISO-induced MI in rats by decreasing the levels of mitochondrial lipid peroxidation products and reinstates normal mitochondrial architecture as well as function. The maintenance of
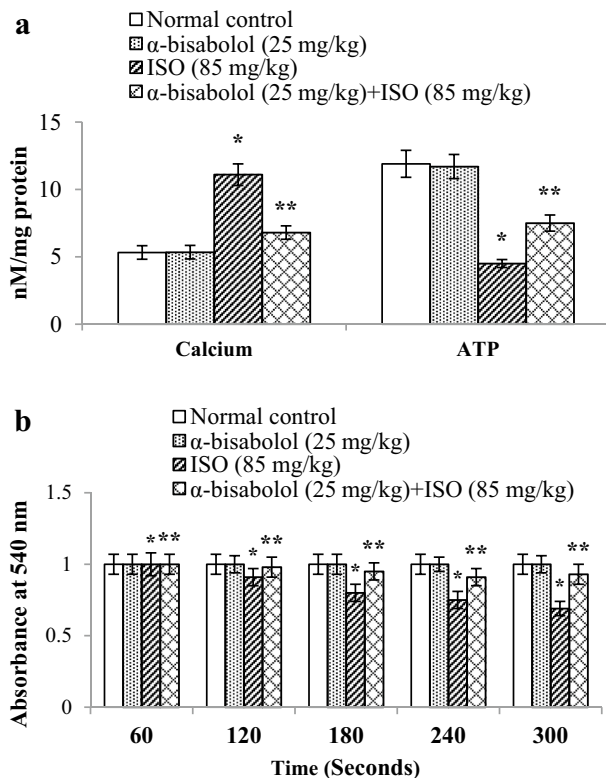
Fig. 4 Measurement of Ca^{2+} homeostasis and energy production in the heart mitochondria. **a** Levels of Ca^{2+} and ATP in the heart mitochondria. **b** Mitochondrial swelling assay. Each column is mean $\pm$ SD for eight rats in each group; $*P < 0.05$ as compared to normal control (Group-I), $**P < 0.05$ as compared to ISO control (Group-III) (DMRT)

mitochondrial lipid peroxide levels reasonably ascribed to the free radical scavenging activity of α-bisabolol.

Impairment in the efficiency of antioxidants leads to enhanced deleterious effects of ROS exerted through the quinine metabolites of ISO and interferes with antioxidant systems (SOD, catalase and GSH) results in the decreased activities and levels of these antioxidants. Glutathione protects the mitochondrial membrane by attenuating lipid peroxidation and its decreased concentrations seem to be a crucial mechanism for imbalanced mitochondrial function [48]. Increased intracellular GSH content may prevent cellular and mitochondrial damage. α-Bisabolol treatment protects the mitochondria by increasing the mitochondrial antioxidant status and counters ROS-mediated oxidative stress in ISO-induced myocardial infarcted rats by virtue of its potent free radical scavenging property.

ICDH is the predominant enzyme involved in glutathione regeneration by producing reduced nicotinamide adenine dinucleotide phosphate (NADPH) in the heart mitochondria [49]. SDH regulates mitochondrial ATP production whereas MDH present in outer mitochondrial membrane and both are vulnerable to free radical attack [50, 51]. Conversion of α-ketoglutarate to succinyl-CoA and reduced nicotinamide adenine dinucleotide (NADH) in the heart mitochondria is catalyzed by α-KGDH. The aerobic oxidation of pyruvate might be the reason for the reduced activities of TCA cycle enzymes which results in declined ATP production in ISO-induced rats. During free radical triggered hypoxic conditions, the activities of TCA enzymes were declined in ISO-induced rat's heart mitochondria [50]. Previous reports have showed that the inner mitochondrial respiratory chain was inhibited during cardiac damage [52]. Decreased activities of inner mitochondrial respiratory chain enzymes play a crucial role in the progression of left ventricular remodeling and heart failure during MI [53]. It might be due to the unavailability of cardiolipin which is required for their functioning results in the decreased production of ATP in ISO-induced rats [53, 54]. The oxidative damage during MI could have created blocks in the flow of electrons along the respiratory chain. The impairment of mitochondrial complexs might increase the leakage of electrons from the ETC and producing damage to the mitochondrial inner membrane constituents [54, 55]. In our study, the complexes I–IV activities were significantly decreased in the ISO challenged rats compared to normal control rats. α-Bisabolol pre and co-treatment significantly enhanced the activities of these mitochondrial ETC complexes by virtue of its potent free radical scavenging property.

The Ca^{2+} homeostasis in the mitochondria is mandatory for the physiological functions and cell survival. Also, Ca^{2+} is a regulator and the key second messenger for the function of the mitochondria. Excessive ROS may cause extensive oxidative damage which results in impaired cardiomyocyte functions including ion transport, contractility and Ca^{2+} cycling [56]. During ischemia, the depleted ATP levels caused loss of ionic gradients, swelling and alterations in the structure and function of the mitochondria [57]. Swelling of mitochondria is found in line with mitochondrial dysfunction and damage [58]. ISO triggers the mitochondrial permeability transition pore opening which disrupts permeability barrier of the inner mitochondria, which results in uncoupling of oxidative phosphorylation, osmotic swelling, and rupture of the outer membrane and ultimately cell death [51]. α-Bisabolol treatment decreased the mitochondrial Ca^{2+} overload, swelling and increased the ATP levels thereby maintaining the structure and function of the mitochondria.

Apoptosis is one of the inevitable contributors of the cardiac damage [59]. It has been implicated as a possible mechanism in the progress of heart failure due to its pathophysiological consequences contributing to the loss and functional
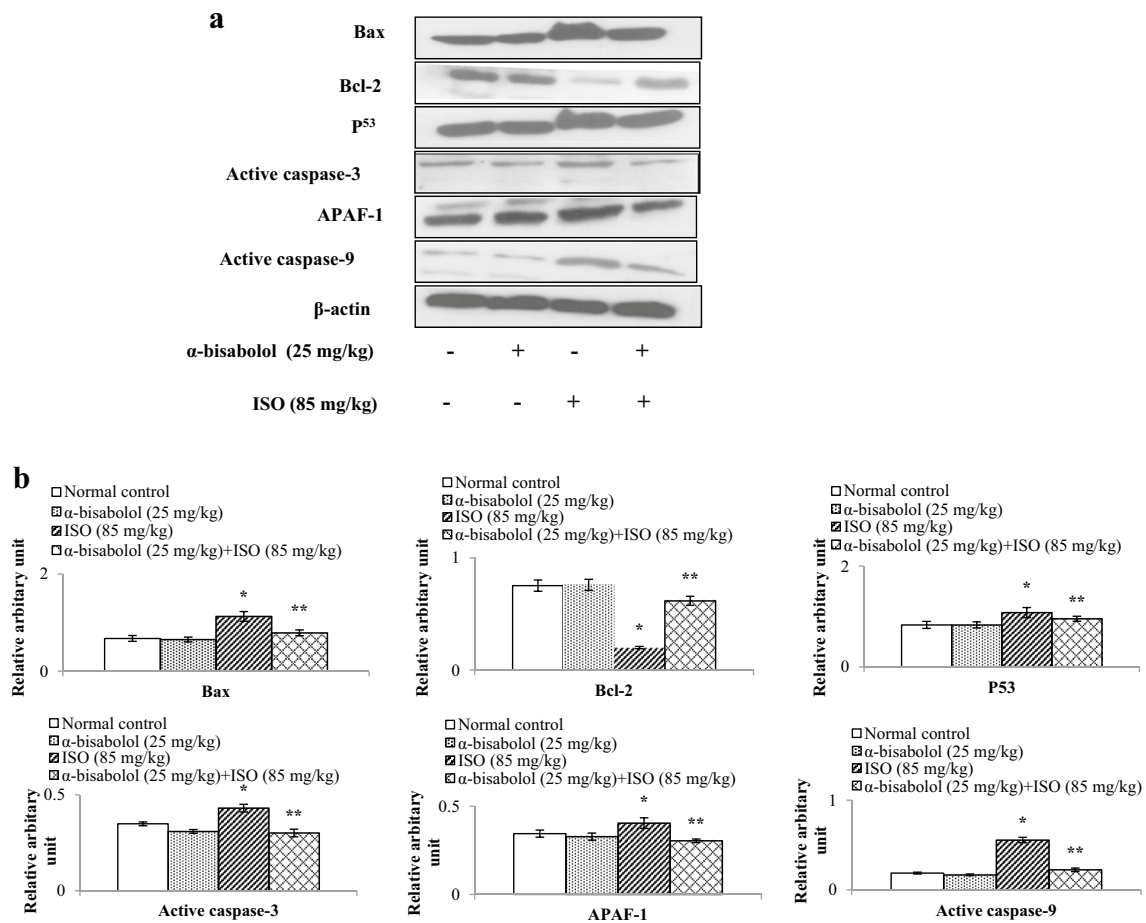
Fig. 5 Western blot analysis of apoptotic proteins. **a** Effect of α-bisabolol on the myocardial protein expressions of Bax, Bcl2, P[53], APAF-1, active caspase-3 and active caspase-9. **b** Quantification of western blots assayed for various apoptotic markers

abnormalities of the cardiac muscle [60]. The β1-adrenergic receptor activation in cardiomyocytes triggers excessive apoptosis by increasing the myocardial contractility which consequently produces hypoxia followed by ischaemia [12]. ISO, a potent β-adrenergic receptor agonist administration has been reported to induce cardiomyocyte death by activating apoptosis in the myocardium [60, 61]. The members of the Bcl-2 family possess crucial role in the progress of apoptosis as evidenced by various experimental studies. The proteins of Bcl-2 family and caspases are checkpoints of the apoptotic pathways [61]. Bax, a proapoptotic gene of the family usually resides in the cytosol is expressed abundantly during apoptosis and promotes the programmed cell death [62]. Increased level of ROS activates Bax which ensures cell death by creating mitochondrial pore formation giving rise to the release of mitochondrial cytochrome-C to the cytosol. On the other side, this process can be countered by anti-apoptotic protein Bcl-2 [63]. Released cytochrome c binds to APAF-1,

a key regulator of mitochondrial intrinsic pathway of apoptosis and activates executioner caspases by the formation of apoptosome [61]. The apoptosome then activates the initiator caspase 9, which subsequently leads to the activation of downstream executioner caspases, such as caspase 3 [64]. P[53] is a regulator of cell cycle progression and apoptosis have been revealed to regulate APAF-1 in response to severe stress conditions and it also possesses the ability to activate pro apoptotic Bax protein [65]. ISO is an abundant producer of ROS and it treatment showed upregulated expressions of Bax, P[53], APAF-1, active caspase-3 and active caspase-9 followed by downregulated expression of Bcl-2. Futhermore, ISO treatment showed decreased expression of cytochrome-C in the mitochondrial fraction and increased expression in the cytosolic fraction. These results have clearly revealed the activation of intrinsic apoptotic pathway in the myocardium. α-Bisabolol administration prevented ISO-induced overexpression of Bax, P[53], APAF-1, active caspase-3 and active

Fig. 6 Western blot analysis for cytochrome-C release. **a** Effect of α-bisabolol on cytochrome-C in the mitochondria and cytosol. **b** Quantification of western blots assayed for cytochrome-C release
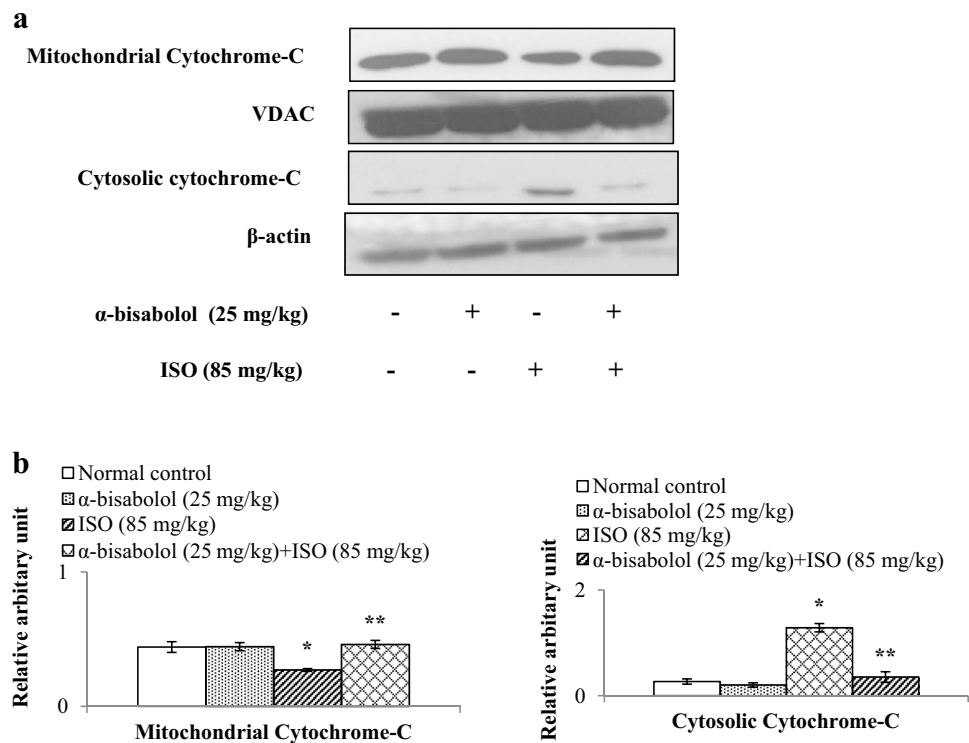

Fig. 7 TEM study on heart mitochondria. **a** Rats treated with α-bisabolol showing normal mitochondria with intact myofibrils (Group-II) (×5000). **b** ISO-induced rats showing loss of cristae (↑), swelling of mitochondria, irrugular shape and size (→), vacuolation (←) with damaged myofibrils (↓) (Group-III) (×5000). **c** α-Bisabolol pre and co-treated ISO-induced rats showed near normal architecture of mitochondria with intact myofibrils (Group-IV) (×5000)
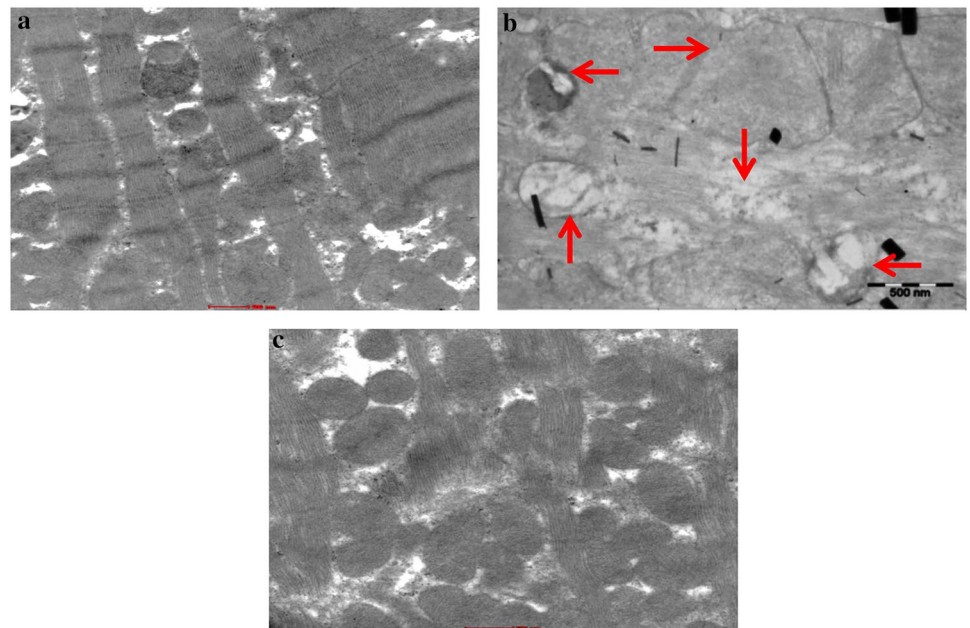
caspase-9. Also, it decreased the release of mitochondrial cytochrome-C from the myocardium. Therefore, α-bisabolol suppresses the activation of mitochondrial pathway of apoptosis in the myocardium.

Ultrastructural alterations of mitochondria are the most prominent finding to reveal cardiac damage in ISO-induced MI. ISO-induced heart mitochondria showed irregular size and shape, vacuolation, swelling and distruption of cristae.

α-Bisabolol pre and cotreatment revealed normal mitochondrial architecture without distruption of cristae, swelling and vacuolation. Thus, α-bisabolol protected the structure of heart mitochondria against ISO-induced damage. The radical scavenging activities are crucial in biological systems due to the role of free radicals in triggering lipid peroxidation [66]. ISO is an abundant producer of OH• which possess the ability to alter the normal function of cell membranes via lipid

OH• scavenging activity of α-bisabolol
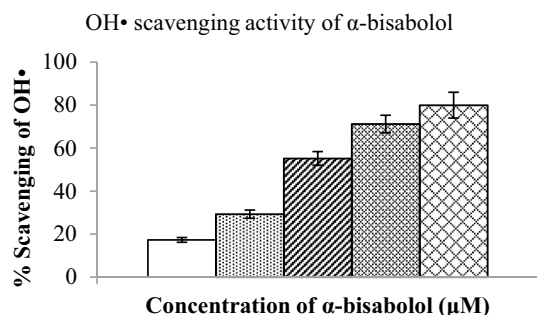
Fig. 8 The in vitro free radical scavenging effects of α-bisabolol on OH$^\bullet$. Columns are the average of triplicate experiments

peroxidation. In our study, α-bisabolol scavenges OH$^\bullet$ in a dose-dependent manner. The highest percentage scavenging of OH$^\bullet$ was observed at the concentration of α-bisabolol (50 μM). Thus, α-bisabolol scavenges excess OH$^\bullet$ produced by the metabolism of ISO and attenuates cardiac mitochondrial lipid peroxidation by virtue of its free radical scavenging capacity.

The overall protective effects of α-bisabolol seems to be closely involved with the restriction of cardiac marker leakage, decreasing mitochondrial oxidative stress, maintaining TCA cycle enzymes, ETC complexes, Ca^{2+} homeostasis and structure of heart mitochondria and standing against the mitochondrial pathway of apoptosis.

Conclusion

Our findings revealed that α-bisabolol has maintained cardiac mitochondrial structure and function in ISO-induced MI in rats. Taken together the biochemical parameters, transmission electron microscopic, molecular and in vitro studies clearly suggest the therapeutic potential of α-bisabolol in attenuating myocardial injury. However, the translation of these therapeutic benefits in humans require many further studies. The possible mechanisms for the observed protective effects of α-bisabolol are ascribed to the prevention of oxidative stress triggered mitochondrial dysfunction and intrinsic apoptotic pathway in IS-induced MI in rats.

Compliance with ethical standards

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