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ORIGINAL ARTICLE

Prophylactic Treatment with Icariin Prevents Isoproterenol-Induced Myocardial Oxidative Stress via Nuclear Factor-Like 2 Activation

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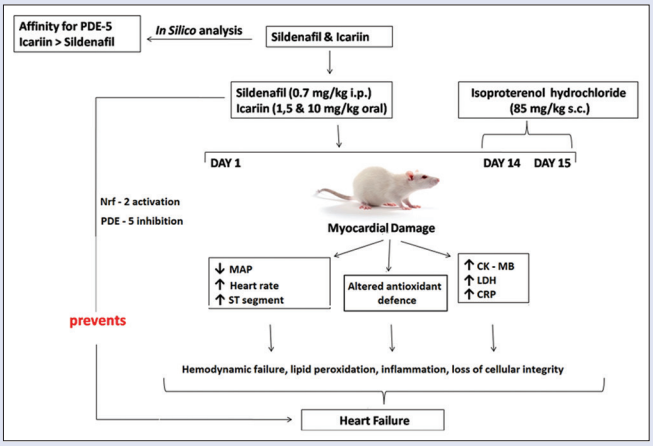
ABSTRACT

Introduction: Icariin, a major component of *Epimedium* species and a mild phosphodiesterase 5 (PDE-5) inhibitor, was evaluated for the prevention of myocardial oxidative stress in isoproterenol (ISO)-challenged Wistar rats. **Objective:** This study aimed to evaluate the cardioprotective action of icariin in ISO-intoxicated rats. **Materials and Methods:** Rats were daily treated with icariin (1, 5, and 10 mg/kg, p.o.) and sildenafil (0.7 mg/kg, i.p.) for 15 days. Oxidative stress was induced by subcutaneous administration of ISO (85 mg/kg s.c) in two consecutive doses at an interval of 24 h on 14th and 15th day of the study. After induction, rats were anesthetized for recording the electrocardiogram (ECG) and then sacrificed to perform immunohistochemistry and biochemical assays of heart tissue. **Results:** ISO treatment resulted in a marked increase in lipid peroxidation, serum markers (lactate dehydrogenase [LDH], creatine kinase-MB [CK-MB], and C-reactive protein [CRP]), and infarct size and a significant decrease in the level of reduced glutathione (GSH) and endogenous antioxidant enzymes in the myocardium. Lowering of arterial blood pressure and alteration in ECG showed significant alteration in cardiac hemodynamics. Hematoxylin and eosin staining of the cardiac tissue showed considerable myocardial damage. Pretreatment with icariin (5 and 10 mg/kg, p.o.) and sildenafil (0.7 mg/kg, i.p) significantly decreased the elevated lipid peroxidation, LDH, CK-MB, and CRP. Moreover, the results also showed an increase in endogenous antioxidants and protein expression of nuclear factor-like 2 (Nrf-2) when compared to the ISO-treated group. **Conclusion:** The results indicated that icariin significantly ameliorates the ISO-induced oxidative stress and restores membrane integrity and cellular damage. Thus, we can conclude that the activation of Nrf-2 signaling and PDE-5 inhibition by icariin is possibly responsible for the cardioprotection.

Key words: *Epimedium* species, icariin, isoproterenol, nuclear factor-like 2, oxidative stress, phosphodiesterase 5 inhibitor, sildenafil citrate

SUMMARY

- Icariin is a major constituent of *Epimedium* herb which is traditionally used in cardiovascular diseases for years
- Isoproterenol (ISO)-induced cardiac injury mimics human myocardial infarction
- Icariin was evaluated for its cardioprotective role in ISO-intoxicated rats
- Icariin proved to be effective in the amelioration of oxidative stress caused by ISO administration in rats
- Activation of nuclear factor-like 2 signaling and phosphodiesterase 5 inhibition are collectively responsible for its cardioprotective effects.



Abbreviations used: Nrf-2: Nuclear factor-like 2, PDE-5: Phosphodiesterase-5, ISO: Isoproterenol, TCM: Traditional Chinese Medicine, ECG: Electrocardiogram, LDH: Lactate dehydrogenase, CK-MB: Creatine kinase-MB, CRP: C-reactive protein, GSH: Endogenous glutathione, TBARS: Thiobarbituric acid reactive substances, GPx: Glutathione peroxidase, GR: Glutathione reductase, GST: Glutathione-S-transferase, SOD: Superoxide dismutase, CAT: Catalase, H and E: Hemotoxylin and eosin, MAPK: Mitogen-activated protein kinase, CPCSEA: Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines, DMSO: Dimethyl sulfoxide, MM-GBSA: Molecular mechanics-generalized born surface area, BPM: Beats per minute.

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INTRODUCTION

Heart failure is a major public health problem affecting 26 million people worldwide. Higher prevalence of heart failure is contributing significant losses to the health carers in the form of productive lives and costs of treatment.[1] The pathology of heart failure is very complicated and characterized by multiple etiologies. However, the patients having a history of myocardial ischemia (MI) are most vulnerable to develop heart failure. Oxidative stress is one such contributing factor that leads to the development of post-MI heart failure.[2]

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Isoproterenol (ISO) hydrochloride, a synthetic catecholamine, and β-adrenergic agonist cause severe oxidative stress in the myocardium.[3] Augmented oxidative stress leads to the lipid peroxidation, increased membrane permeability, and sarcolemmal Ca^{2+} overload.[4] ISO also increases the plasma renin activity that further activates the renin-angiotensin system and contributes to heart failure.[5] Furthermore, excess reactive oxygen species (ROS) production leads to the necrosis of cardiac tissue. The pathophysiological changes produced by ISO in rat hearts are comparable to those observed in human myocardial infarction.[6]

Phosphodiesterase-5 (PDE-5) inhibitors are well known for their important role in the treatment of erectile dysfunction and pulmonary hypertension. Nowadays, PDE-5 inhibitors are extensively reviewed for their new indications. Small clinical trials and animal studies conducted showed their probable role in heart failure and associated manifestations.[7] Sildenafil, a prototype of PDE-5 inhibitor, has been reported to have antihypertrophic activity in rats via modulation of cGMP and its kinases,[8] opening of K+ channels,[9] and reversal of fibrosis.[10]

Icariin [Figure 1] is an important constituent of *Epimedium* species (*Berberidaceae*), which is widely used in the Traditional Chinese Medicine for sexual dysfunction, osteoporosis, and heart failure.[11] In addition, icariin has shown antihypertrophic effect via inhibiting matrix metalloproteinase (MMP)-2 and MMP-9 in ISO-intoxicated rats.[12] The importance of icariin in oxidative stress is recently established by an *in vitro* study, showing the involvement of mitogen-activated protein kinase activation.[13] *In vivo* examination of icariin in an established oxidative stress model has yet not explored.

In the present study, we compared the affinities of icariin and sildenafil for PDE-5 receptor by molecular docking. Further, the cardioprotective potential of icariin and sildenafil in ISO-induced oxidative stress was evaluated in rodents. Results from the *in silico* and *in vivo* studies were correlated to elucidate the mechanism involved.

MATERIALS AND METHODS

Experimental animals

All the experimental procedures involving the use of experimental animals were approved by the Institutional Animal Ethics Committee (protocol number 1042/2015, approval date: 03/03/2014), Hamdard University (India) by the Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines. Wistar albino male rats weighing 200–250 g were procured from the Central Animal House Facility, Hamdard University, New Delhi (India). The animals were housed in polypropylene cages for 1 week to acclimatize with the standard conditions (12-h light: dark cycle; temperature, 23°C ± 2°C; relative humidity, 60% ± 5%). The standard pellet diet and free access to the tap water were provided to the animals.

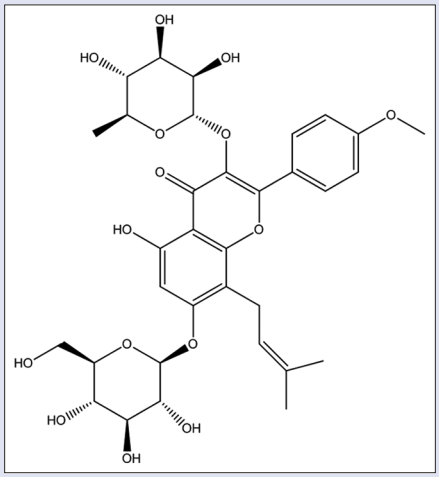

Figure 1: Chemical structure of icariin

Drugs and reagents

Icariin (purity >97% by high-performance liquid chromatography [HPLC]) and ISO hydrochloride (purity >98%) were purchased from Cayman Chemicals and TCI Chemicals, respectively. Sildenafil citrate (purity >98%) was procured from Pfizer Ltd. Triphenyl tetrazolium chloride (TTC, purity >98%), urethane (purity >98%), colorimetric kits for C-reactive protein (CRP), lactate dehydrogenase (LDH), and creatine kinase-MB (CK-MB) were purchased from CDH, Chemsworth, Span diagnostics, and Reckon Diagnostics Pvt. Ltd, respectively. The antibody used for immunohistochemistry was brought from SantaCruz Biotechnology (nuclear factor-like 2 [Nrf-2] antibody). All other chemicals used in biochemical estimations were of analytical grade. HPLC grade water was used for all biochemical analysis.

Experimental design

After 1 week of acclimatization, Wistar rats were randomly divided into eight groups ($n = 6$) and treated as follows:

- Vehicle control: Administered vehicle (0.1% dimethyl sulfoxide [DMSO] in phosphate buffer solution) orally for 15 days and 0.1 ml saline s.c. on the 14th and 15th day of the treatment
- ISO-treated: Administered vehicle (0.1% DMSO in phosphate buffer solution) orally for 15 days and ISO (85 mg/kg s.c.) on the 14th and 15th day of the treatment
- Icariin *per se*: Administered 10 mg/kg of icariin orally for 15 days
- Sildenafil *per se*: Administered 0.7 mg/kg of sildenafil citrate (i.p.) for 15 days
- Icariin 1 + ISO: Administered 1 mg/kg of icariin orally for 15 days and ISO (85 mg/kg s.c.) on the 14th and 15th day of the treatment
- Icariin 5 + ISO: Administered 5 mg/kg of icariin orally for 15 days and ISO (85 mg/kg s.c.) on the 14th and 15th day of the treatment
- Icariin 10 + ISO: Administered 10 mg/kg of icariin orally for 15 days and ISO (85 mg/kg s.c.) on the 14th and 15th day of the treatment
- Sildenafil + ISO: Administered sildenafil citrate 0.7 mg/kg, i.p for 15 days and ISO (85 mg/kg s.c.) on the 14th and 15th day of the treatment.

In silico analysis

To establish possible binding modes of icariin to receptor and good biological activity on the basis of structures, molecular docking studies were carried out using Glide Extra Precision (XP) Maestro 10.1 Schrodinger, running on Linux 64 operating system based on X-ray crystal structure of key enzyme that are important for cardiovascular process including PDE-5. The structure of PDE-5 retrieved from protein data bank (www.rcsb.org) (PDB: 1UDT). Molecular docking studies mainly involve selection and preparation of appropriate protein, grid generation, ligand preparation followed by docking and its analysis. The docking score and hydrogen bonds and pi–pi interaction formed with the surrounding amino acids are used to predict their binding affinities and proper alignment of these compounds at the active site of the enzyme. Binding energy estimation can be used to calculate ligand binding energies and ligand strain energies for a set of ligands and a single receptor using the prime molecular mechanics-generalized born surface area (MM-GBSA) method, Maestro 10.1. The more energetically favorable conformation was selected as the best pose.

Blood pressure and electrocardiographic measurement

After 24 h of the last dose, animals were weighed and anesthetized with urethane (1 g/kg, i.p.) to record the arterial blood pressure. A micromanometer-tipped catheter (Millar Instruments) was used to record the systolic and diastolic arterial pressure in the femoral artery. For electrocardiogram (ECG) recording, the standard limb lead II of the surface ECG was used for each rat by the PowerLab Data Acquisition System (AD Instruments, Australia) connected to a computer enabled with LabChart professional software version 8 (AD Instruments, Australia). The ECG record exhibits the following parameters: heart rate in beats per minute, ST height, RR, and QRS complex. After completion of blood pressure and ECG studies, blood was collected, centrifuged to separate serum, and stored at −20°C ± 5°C for biochemical assays. The heart was removed, washed in ice-cold normal saline, soaked by tissue paper, and then weighed. A small piece of heart's left ventricle was preserved in formalin solution (10%) for histopathology, immunohistochemistry, and infarct area estimation. Remaining major pieces of the heart were preserved at −20°C ± 5°C for biochemical studies.

Biochemical estimations in serum

Activities of CK-MB,[14] LDH,[15] and CRP[15] were estimated in the serum using commercially available kits.

Biochemical estimations in myocardial tissue

The level of thiobarbituric acid reactive substances (TBARS), reduced glutathione (GSH), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), superoxide dismutase (SOD), and catalase (CAT) were estimated by the methods published earlier.[16-22]

Immunohistochemistry of myocardial tissue

Paraffin sections (5 µm thick) were deparaffinized in xylene and rehydrated with a graded series of ethanol followed by washing under running double distilled water. Sections were subjected to antigen retrieval using citrate buffer (pH 6). Sections were cooled at room temperature for 10 min, incubated in 4% hydrogen peroxide for 15 min to remove the background staining, and then washed for three times using tris-buffered saline. Next, the sections were incubated overnight at 4°C with the primary Nrf-2 antibody (1:100, SantaCruz Biotechnology). Sections were rinsed with buffer and incubated for 1 h with peroxidase-conjugated secondary antibody. The reaction was visualized using diaminobenzidine solution. Photomicrographs were taken with a computer-enabled Meiji Microscope. Fiji (Image J) software was used for the semi-quantification of the protein expression by reciprocal intensity method. The range of pixel intensities of images was between 0 and 250. Values 0 and 250 indicate the darkest and the lightest shade of image color, respectively.[23]

Estimation of infarcted area

Rat's hearts were sectioned into six slices each with 2-mm thickness and immersed in 2% TTC dye at 37°C for 30 min. The heart was differentiated according to white-colored infarct area and red-purple noninfarct area. The slices were placed on a glass plate in a row, and the images were captured using a digital camera. Image J Software version 1.49 (NIH, Bethesda, MD, USA) was used for the measurement of infarction at constant saturation and brightness of 188 and 125, respectively. The percentage of the area of infarction was calculated as ([white or yellow area/total area] × 100).[24]

Histopathology

Myocardial tissue was fixed in 10% formalin immediately after the sacrifice, processed, and impregnated with paraffin. Paraffin sections were cut and stained with hemotoxylin and eosin (H and E) dye. Photomicrographs were taken using Meiji fluorescent microscope enabled with lumenera camera. The images were analyzed with Infinity Analyze 3 Software (Lumenera Corp., Canada).

Data presentation and statistical analysis

Statistical analyses were carried out using Prism software package version 7 (GraphPad Software Inc., California, USA). Data are expressed as the mean ± standard error of the mean. One-way analysis of variance was carried out, and the statistical comparisons among the groups were performed with Tukey's test. $P < 0.05$ was considered statistically significant.

RESULTS

In silico comparison of icariin and sildenafil

The compound icariin is well fitted in the active sites of PDE-5. The titled compound icariin studied for PDE-5 was found to be most potent and has high docking score of −15.915 as compared to sildenafil which has docking score of −12.390 [Table 1]. The icariin also assumes favorable orientation within the PDE-5 binding sites. The docked pose of the icariin observed nine hydrogen bond interactions with amino acid TYR 664 (OH…O, 1.88 Å), LEU 725 (O…HN, 2.47 Å), SER 663 (OH…O = C, 1.89 Å), THR 723 (OH…O = C, 2.24 Å), GLU 682 (OH…O = C, 2.82 Å and OH…O = C, 2.45 Å), ASP 654 (OH…O, 2.33 Å), and HIS 613 (O…NH, 1.86 Å and O…NH, 2.68 Å) at the binding site of PDE-5 [Figure 2]. Interestingly, icariin also forms a pi–pi interaction between pyrone ring and phenyl ring with PHE 786, whereas sildenafil forms hydrogen bonds with SER 663, GLN 817, and TYR 612 and pi–pi interaction with PHE 820 and TYR 612 [Figure 2]. The dG binding energy of the compounds icariin and sildenafil was calculated by Prime MM-GBSA, Maestro 10.1, and found to be −91.515 kcal/mol and −71.413 kcal/mol, respectively, for PDE-5. This result justifies that the icariin has a high affinity for PDE-5 than sildenafil [Table 1].

Effect of icariin and sildenafil on blood pressure

Table 2 shows the alteration in arterial blood pressure in various groups. Rats treated with ISO showed a significant decrease in the

Table 1: Binding affinity of icariin and sildenafil for phosphodiesterase-5 enzyme

Compound	Icariin	Sildenafil
Docking score	−15.915	−12.390
Hydrogen bonding	9	3
Pi–Pi interactions	Yes	Yes
dG binding energy	-95.515 kcal/mol	-71.413 kcal/mol

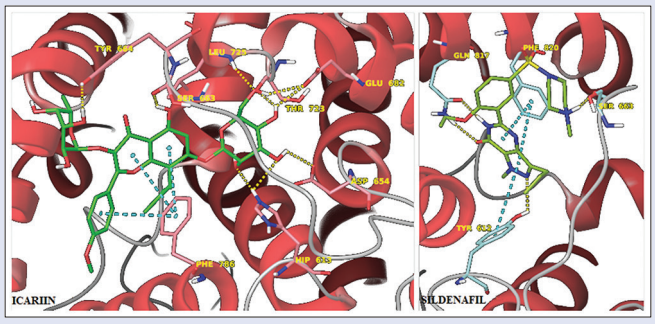

Figure 2: *In silico* analysis of icariin and sildenafil showing binding with phosphodiesterase-5 enzyme

systolic, diastolic, and mean arterial pressures ($P < 0.001$, $P < 0.05$, and $P < 0.001$, respectively). Pretreatment with sildenafil (0.7 mg/kg/day, i.p.) significantly increased the systolic, diastolic, and mean arterial blood pressure as compared to ISO-treated group ($P < 0.001$). Pretreatment with icariin (10 mg/kg/day, p.o.) significantly increased the systolic, diastolic, and mean arterial blood pressure in comparison to the ISO-treated group ($P < 0.01$). Pretreatment with the lower and medium doses of icariin (1 mg/kg/day and 5 mg/kg/day, p.o.), however, nonsignificantly altered the blood pressure as compared to the ISO-treated group.

Effect of icariin and sildenafil on electrocardiological changes

In addition to arterial blood pressures, substantial changes in the ECG have also been observed after ISO treatment [Figure 3]. The control rats showed a typical ECG pattern, whereas ISO-treated rats showed an increase in heart rate ($P < 0.001$), elevated ST segment ($P < 001$), merged T and P waves, flutter waves, decreased RR interval ($P < 0.01$), and widening of QRS complex ($P < 0.001$). Pretreatment with icariin (5 mg/kg/day and 10 mg/kg/day) and sildenafil (0.7 mg/kg/day, i.p.) substantially lowered the heart rate and ST segment elevation and reversed the changes in RR interval and QRS complex. Lower dose of icariin (1 mg/kg/day) showed nonsignificant effects in comparison to the ISO-treated group [Table 2].

Effect of icariin and sildenafil on lactate dehydrogenase, creatine kinase-MB, and C-reactive protein levels

Table 3 represents the effect of icariin and sildenafil on the levels of serum CK-MB, LDH, and CRP of various groups. The rats treated with ISO showed a noticeable increase of LDH, CK-MB, and CRP ($P < 0.001$, each) in comparison to the control ($P < 0.001$). Pretreatment with sildenafil and high dose of icariin significantly decreased the level of

Table 2: Effects of icariin and sildenafil on isoproterenol-induced blood pressure and electrocardiographic changes

Groups	SAP (mmHg)	DAP (mmHg)	MAP (mmHg)	Heart rate (BPM)	ST height (mv)	RR interval (s)	QRS complex (s)
Vehicle control	135.3±5.22	98.17±6.04	110.6±5.04	274.9±11.91	0.35±0.005	0.21±0.001	0.019±0.001
Isoproterenol treated	98.17±6.04***	68.50±2.94*	78.39±1.99**	347.8±15.18*	0.64±0.014***	0.13±0.007**	0.024±0.001***
Icariin *per se*	124.5±3.50	92.17±3.61	102.9±1.74	302.7±9.61	0.30±0.013	0.22±0.001	0.018±0.001
Sildenafil *per se*	102.8±0.16	77.67±7.80	86.06±5.18	255.4±0.20	0.39±0.006	0.25±0.036	0.017±0.001
Icariin 1 + ISO	112.6±7.90	76.17±8.30	89.50±7.34	326.3±12.80	0.61±0.006	0.15±0.003	0.026±0.001
Icariin 5 + ISO	110±6.61	79.83±7.14	89.89±5.69	281.3±23.14#	0.58±0.003##	0.24±0.004###	0.016±0.001###
Icariin 10 + ISO	125.2±4.87##	85.67±8.39	98.83±7.02	261.1±13.63##	0.40±0.020###	0.23±0.001###	0.014±0.001###
Sildenafil + ISO	135.8±1.75###	101±3.79#	112.6±3.02###	261.7±13.60##	0.39±0.011###	0.22±0.001##	0.016±0.001###

Results are expressed as mean±SEM. (n=6). *P<0.05, **P<0.01, ***P<0.001 versus vehicle control group. #P<0.05, ##P<0.01, ###P<0.001 versus isoproterenol-treated group. SEM: Standard error of mean; ISO: Isoproterenol; MAP: Mean arterial pressure; SAP: Systolic arterial pressure; DAP: Diastolic arterial pressure

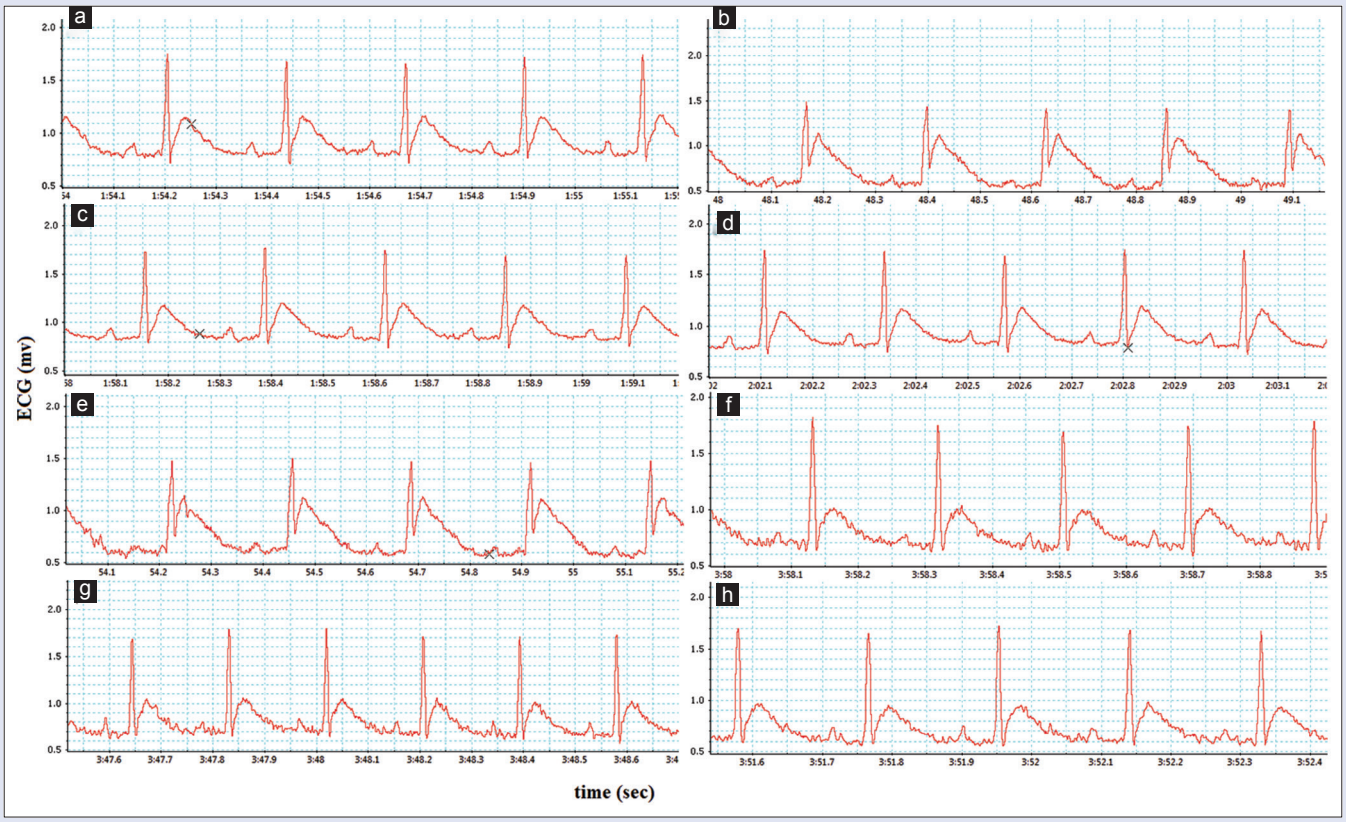

Figure 3: Effect of icariin and sildenafil on isoproterenol-induced electrocardiographic changes. (a) Vehicle control, (b) isoproterenol treated, (c) icariin *per se*, (d) sildenafil *per se*, (e) icariin 1+ isoproterenol, (f) icariin 5+ isoproterenol, (g) icariin 10+ isoproterenol, (h) sildenafil + isoproterenol

CK-MB, LDH, and CRP ($P < 0.001$). Pretreatment with medium dose of icariin (5 mg/kg/day, p.o.) also decreased the level of CK-MB, LDH, and CRP but to a lesser extent ($P < 0.05$ for LDH, $P < 0.01$ for CK-MB, $P < 0.001$ for CRP). However, icariin at a lower dose (1 mg/kg/day, p.o.) did not show any significant difference when compared with the toxic group.

Effect of icariin and sildenafil on lipid peroxidation and antioxidant enzymes

The myocardial TBARS and GSH levels and activities of SOD, CAT, GPx, GR, and GST in the heart of various treatment groups are shown in Figures 4 and 5. The ISO-treated group showed a significant increase in the level of TBARS ($P < 0.001$) in the cardiac tissue as compared to the normal control rats. On the contrary, ISO treatment substantially decreased the levels of endogenous GSH, SOD, CAT, GPx, GR, and GST ($P < 0.001$). Pretreatment with sildenafil (0.7 mg/kg/day, i.p.) and icariin (10 mg/kg/day and 5 mg/kg/day, p.o.) in ISO-treated rats significantly prevented the deteriorated antioxidant defense ($P < 0.001$, $P < 0.001$, and $P < 0.01$, respectively. However, the lower dose of icariin (1 mg/kg/day, p.o.) found unable to reverse the changes produced by ISO treatment.

Table 3: Effects of icariin and sildenafil on serum levels of lactate dehydrogenase, creatine kinase-MB, and C-reactive protein

Groups	LDH (IU/L)	CK-MB (IU/L)	CRP (mg/L)
Vehicle control	249.48±7.98	183.14±1.83	1.53±0.10
Isoproterenol treated	685.4±3.40***	535.4±6.50***	16.19±0.23***
Icariin *per se*	216.05±1.44	199.41±4.53	1.52±0.12
Sildenafil *per se*	223.75±1.25	181.57±4.49	1.51±0.10
Icariin 1 + ISO	679.66±5.02	529.98±5.15	16.31±0.08
Icariin 5 + ISO	622.74±3.02###	505.39±1.26#	12.23±0.06###
Icariin 10 + ISO	450.75±9.05###	406.32±1.39###	8.7±0.21###
Sildenafil + ISO	292.33±17.43###	249.83±12.67###	5.24±0.08###

Results are expressed as mean±SEM. (n=6). ***P<0.001 versus vehicle control group. #P<0.05, ###P<0.001 versus isoproterenol-treated group. LDH: Lactate dehydrogenase, CK-MB: Creatine kinase-MB, CRP: C-reactive protein; SEM: Standard error of mean; ISO: Isoproterenol

Effect of icariin and sildenafil on protein expression of nuclear factor-like 2

The protein expression of Nrf-2 in the normal and ISO-treated hearts is represented in Figure 6. Immunohistochemical analysis showed that ISO injection significantly increased the expression of Nrf-2 ($P < 0.001$). Further, the pretreatment with icariin (5 mg/kg/day and 10 mg/kg/day) and sildenafil (0.7 mg/kg/day) up-regulated the expression of Nrf-2 ($P < 0.01$, $P < 0.001$ and $P < 0.001$, respectively), when compared to ISO-treated groups.

Effect of icariin and sildenafil on infarct size

The representative images of infarction size as stained TTC are shown in Figure 7. The ISO-treated group showed large unstained area in comparison to control group ($P < 0.001$). However, the heart slice of treatment groups exhibited the dose-dependent restriction of development of infarct area. Pretreatment with icariin, on all doses, and sildenafil significantly reduced the percent infarct are in comparison

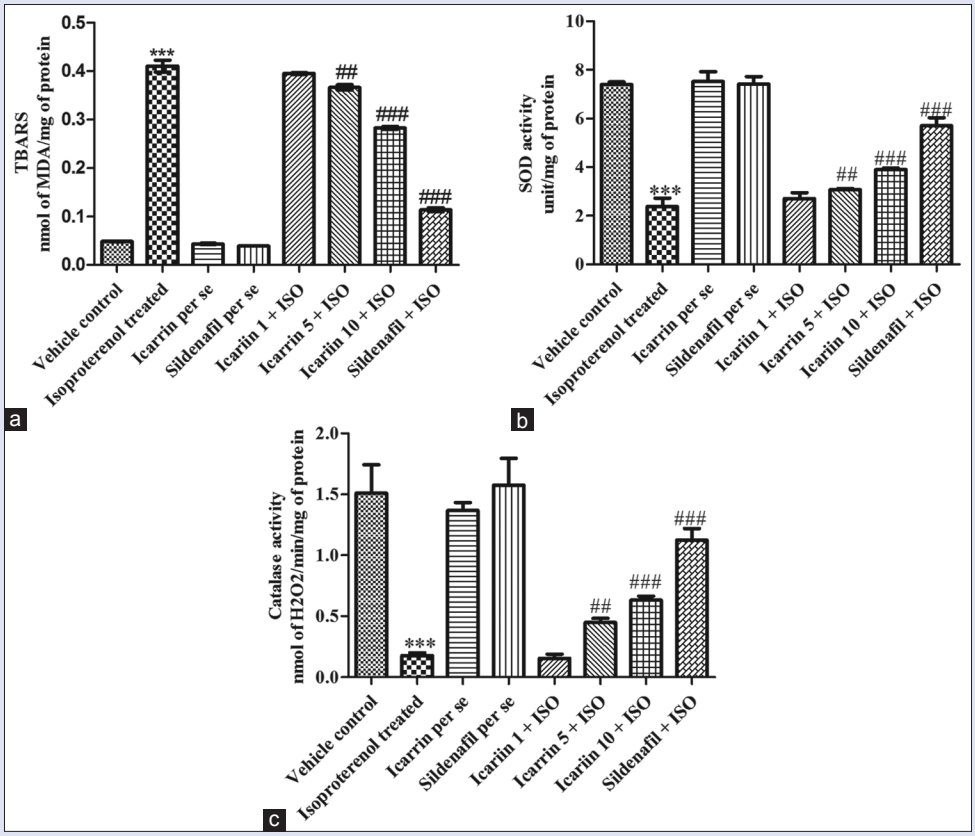

Figure 4: Effect of icariin and sildenafil on isoproterenol-induced changes: (a) Thiobarbituric acid reactive substances, (b) superoxide dismutase, (c) catalase in the myocardium. Values are expressed as mean ± standard error of the mean (n = 6), where ***P < 0.001 versus normal control; ##P < 0.01, and ###P < 0.001 versus isoproterenol treated

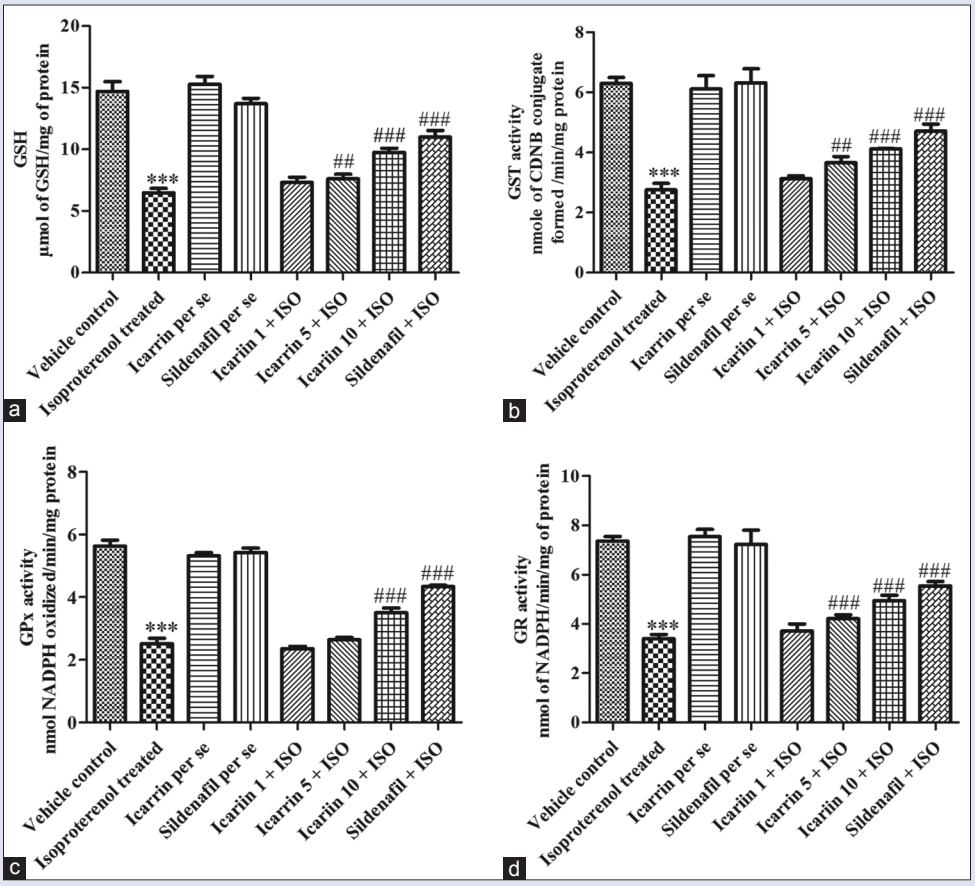

Figure 5: Effect of icariin and sildenafil on isoproterenol-induced changes in myocardium (a) reduced glutathione, (b) glutathione-S-transferase, (c) glutathione peroxidise, (d) glutathione reductase. Values are expressed as mean ± standard error of mean, $n = 6$, where $^{***}P < 0.001$ versus vehicle control; $^{##}P < 0.01$, and $^{###}P < 0.001$ versus isoproterenol treated

to ISO-treated group ($P < 0.001$). *Per se* groups showed no significant infarction as that of the control group.

Effect of icariin and sildenafil on histopathology

Figure 8 and Table 4 shows the histopathological changes in various groups. Control and *per se* groups showed the normal integrity of myocardial tissue with striations, branched appearance, and continuity with adjacent myofibrils. The ISO-treated group showed significant myodegeneration, necrosis of cardiac tissue, pyknotic nucleus, infiltration of macrophages and lymphocytes, and misshapen cell nuclei. Pretreatment with sildenafil and icariin (10 mg/kg/day, p.o.) prevented the necrotic changes and cellular infiltration. Other doses of icariin (1 and 5 mg/kg/day, p.o.) have shown lesser protection as compared to the sildenafil and the higher dose of icariin.

DISCUSSION

ISO is a β-adrenergic agonist which produces oxidative stress via the generation of ROS. Augmented oxidative stress contributes to apoptosis and fibrosis, which eventually leads to the condition mimicking human heart failure.[25,26]

PDE-5 inhibition is a novel approach that has demonstrated its effectiveness in heart failure.[7-9] *In silico* analysis is a modern tool for ascertaining the binding affinity of the substrate with the enzyme at the molecular level. Icariin showed excellent binding with PDE-5 enzyme and was comparable with sildenafil. This encouraged us to evaluate the *in vivo* potential of PDE-5 inhibition by icariin against ISO generated oxidative stress and to elucidate the mechanism of cardioprotection.

Redox-sensitive transcription factor Nrf-2 has been known to regulate the induction of phase 2 detoxifying and antioxidant genes. Nrf-2 signaling has already demonstrated a key role in preventing cardiac injury in both *in vitro* and *in vivo* studies. Nrf-2 mediates the upgradation of antioxidant defense via mitochondrial cell signaling and NADPH oxidase.[27] NADPH oxidase plays a crucial role in generating peroxynitrite and superoxide ions, which leads to oxidative stress. The protective enzymes such as SOD convert the superoxide ions into H_2O_2, which eventually dissociates into water and oxygen by CAT. Endogenous GSH also removes H_2O_2 and gets converted into oxidized glutathione (GSSG) by GPx enzyme. GSSG then gets converted back to GSH using NADPH in the presence of GR enzyme. GST also removes ROS by conjugating itself with GSH and decreasing the oxidative stress.[28] Various studies reported that ISO severely disturbs this antioxidant homeostasis and contributes to the significant cardiac injury.[29,30] We in our study also observed the diminished antioxidant activities in ISO (85 mg/kg, s.c.)-administered rats. It occurred due to the generation of ROS which in turn decreased the level of GSH and endogenous antioxidant enzymes. Pretreatment with icariin and sildenafil restored the antioxidant defense, except the lower dose of icariin. Sildenafil and icariin have already been reported to suppress oxidative stress in various studies.[31,32] Lu *et al.* (2010) showed that oxidative stress increases the expression of PDE-5 in failing heart.[33] Hence, the PDE-5 inhibition in addition to the suppression of oxidative stress may precisely prevent the development of heart failure. Sildenafil

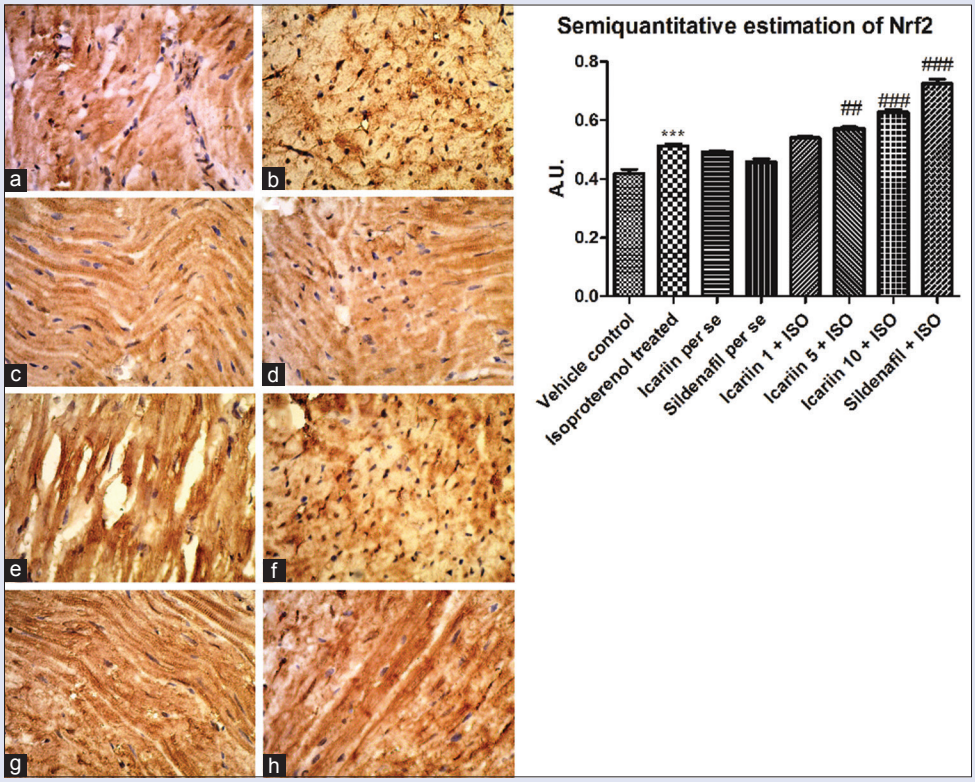

Figure 6: Representative images showing immunohistochemical staining of nuclear factor-like 2 expression in the myocardium. (a) Vehicle control, (b) isoproterenol treated, (c) icariin *per se*, (d) sildenafil *per se*, (e) icariin 1+ isoproterenol, (f) icariin 5+ isoproterenol, (g) icariin 10+ isoproterenol, (h) sildenafil + isoproterenol. Bar graph showing the level of nuclear factor-like 2 activation in myocardium expressed as the optical density. Values are expressed as mean ± standard error of mean ($n = 6$), where $^{***}P < 0.001$ versus normal control; $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ versus isoproterenol treated

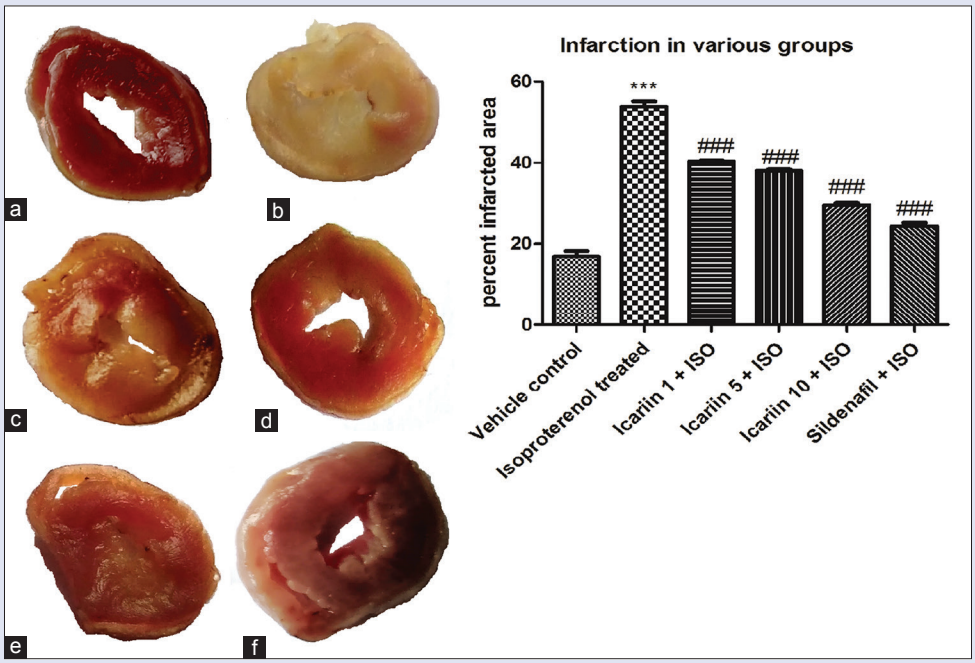

Figure 7: Representative images showing percent infarcted area by TTC staining in myocardium. (a) Vehicle control, (b) isoproterenol treated, (c) icariin *per se*, (d) sildenafil *per se*, (e) icariin 1+ isoproterenol, (f) icariin 5+ isoproterenol, (g) icariin 10+ isoproterenol, (h) sildenafil + isoproterenol. Bar graph showing the percent infarcted area in the myocardium of various group. Values are expressed as mean ± standard error of the mean ($n = 6$), where $^{***}P < 0.001$ versus normal control and $^{\#\#\#}P < 0.001$ versus isoproterenol control

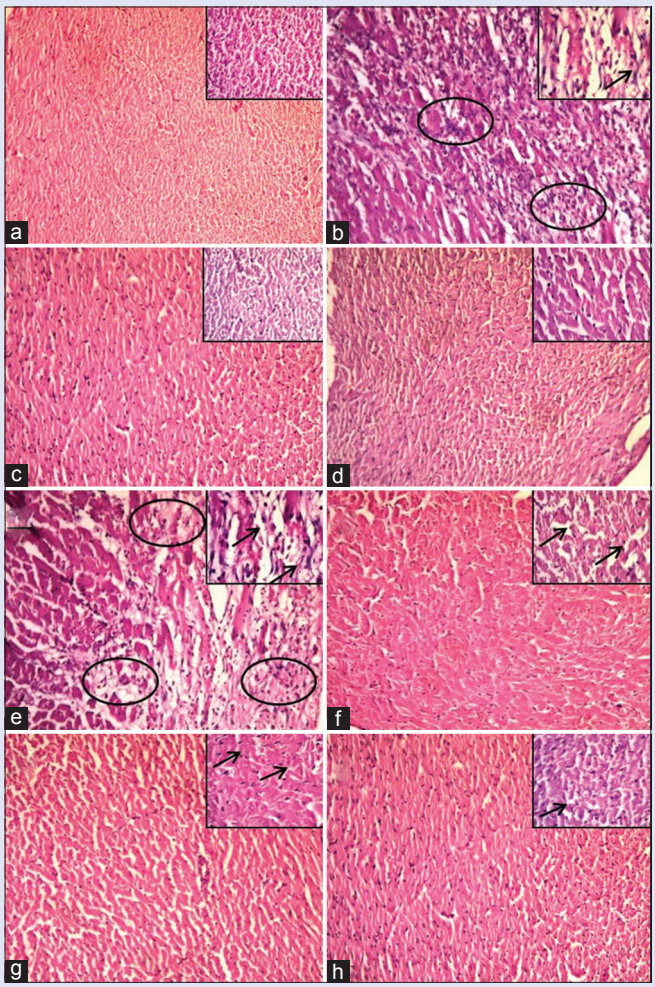

Figure 8: Photomicrographs showing histopathological changes in various groups (×10 and × 40 [inset]). (a) Vehicle control, (b) isoproterenol treated, (c) icariin *per se*, (d) sildenafil *per se*, (e) icariin 1+ isoproterenol, (f) icariin 5+ isoproterenol, (g) icariin 10+ isoproterenol, (h) sildenafil + isoproterenol

has been reported to suppress the NADPH oxidase isoform via PDE-5 inhibition.[34] Icariin in our case probably follows the same course of action.

Treatment with ISO caused lipid peroxidation and increased the malondialdehyde level by 7.5 times in the myocardium, which goes fine with the previous studies that showed a significant increase in TBARS after ISO treatment. Pretreatment with sildenafil and icariin prevented this rise in TBARS. Although the role of PDE-5 inhibition in myocardial lipid peroxidation is unclear, sildenafil and icariin have been reported to show inhibition of lipid peroxidation on different target sites.[35,36] Thus, the possibility of involvement of PDE-5 inhibition in the alleviation of lipid peroxidation cannot be denied.

Blood pressure is an important parameter to assess the dynamics of heart. On treatment with ISO, the systolic, diastolic, and mean arterial blood pressure decreased possibly because of the direct necrosis and decreased calcium ion influx in the myocardium. Our observation is in line with the previous studies that showed fall in blood pressure after ISO exposure.[37] In the present study, pretreatment with sildenafil and icariin prevented this fall in blood pressure as compared to the toxic group. Although the *per se* groups showed a decrease in blood pressure, it is unrelated to toxicity as both sildenafil and icariin cause vasodilatation

Table 4: Effects of icariin and sildenafil on degree of histopathological changes in cardiac tissue

Groups	Inflammatory cells	Altered shape of nucleus	Necrosis	Disorganized myocardial fibers
Vehicle control	0	0	0	0
Isoproterenol treated	+++	++	+++	+++
Icariin *per se*	0	0	0	0
Sildenafil *per se*	0	0	0	0
Icariin 1 + ISO	+++	+	+++	+++
Icariin 5 + ISO	++	0	+	++
Icariin 10 + ISO	0	0	0	0
Sildenafil + ISO	0	0	0	0

The severity of changes was assessed semi-quantitatively and is denoted as follows: 0: No changes; +: Mild changes; ++: Mild changes and +++: Significant changes; ISO: Isoproterenol

and lower the arterial pressure. The maintenance of blood pressure by sildenafil and vardenafil in ischemia-reperfusion injury model is already established.[38] The ethanolic extract of *Epimedium* also reported to maintain the blood pressure in ISO-challenged rats.[12]

Oxidative stress generated by ISO causes loss of cell membrane function which leads to a significant alteration in ECG of Wistar rats. We observed elevated ST segment, merging of P and T wave, flutter waves, increase in the amplitude of P, R, S, and T waves, shorter QRS complex, and decrease in RR interval. Similar findings have also been reported by various previous studies.[39,40] Pretreatment with sildenafil and icariin prevented the ECG changes which showed their membrane-stabilizing property. Previous studies showed that sildenafil has no effect on ECG,[41] so the prevention of deleterious ECG changes is supposedly caused by their antioxidant effect.[42] We are first to report the effects of icariin on ECG changes in ISO-challenged cardiotoxic rats.

C-reactive protein is an important predictor of the inflammation process. Thus, it is a well-accepted biomarker for acute and chronic cardiovascular events.[30] In the present study, CRP of the toxic group has increased 15 folds in comparison to the control group. Pretreatment with sildenafil and icariin significantly prevented the increase in CRP levels. Augmentation of oxidative stress plays a crucial role in the endothelial activation which leads to the release of pro-inflammatory markers such as CRP, tumor necrosis factor-α, and interleukins.[43] Thus, the antioxidant actions of sildenafil and icariin are supposed to be responsible for decreased endothelial activation and release of CRP.

LDH and CK-MB are clinically significant biomarkers used to evaluate the myocardial infarction. Acute injury in the myocardial tissue can easily be detected by the serum levels of these markers.[44] Our study showed steep rise in the serum levels of LDH and CK-MB, indicating severe cellular damage and loss of functional and structural integrity of the cell membrane. These observations are in accordance with the result of previous studies.[30,44] Pretreatment with icariin and sildenafil reduced the generation of oxygen radicals and helped in the preservation of the structural integrity of myocardium.

Furthermore, the administration of ISO can produce an apical dysfunctioning such as left ventricular dysfunction and promote the development of fibrosis.[45,46] This damage can be visualized by TTC staining and measuring infarct size. The infarct area in the heart of toxic

group was found to be significantly large as compared to the control group. We observed that pretreatment with sildenafil and icariin substantially reduced the infarct size. Ockaili *et al.* (2002) reported that rise in cGMP level and the opening of potassium channel through PDE-5 inhibition could decrease the size of infarct produced by ischemia/reperfusion injury.[9] Moreover, the free radical scavenging activity and ability to maintain membrane integrity have also contributed to the decreased infarct size.[47]

Previous studies reported that ISO administration could cause significant damage to the myocardium. H and E staining of the myocardium is an important technique to envisage the extent of injury qualitatively.[48] The toxic group showed the infiltration of macrophages and myodegeneration. However, pretreatment with sildenafil and icariin efficiently prevented the damage done by ISO.

CONCLUSION

PDE-5 inhibition is a novel molecular target for diseases that involve oxidative stress in their pathogenesis. Imbalance in oxidative homeostasis increases the chances of development of more severe form. Some long-term studies with sildenafil showed convincing results in patients with heart failure.[49] Pleiotropic effect of icariin can easily convince the researchers to examine its potency further.[50] Upregulation of protein expression of Nrf-2 contributes to the revival of antioxidant defense mechanism. In concluding remarks, we can say that the activation of Nrf-2 signaling and inhibition of PDE-5 by our drugs (icariin and sildenafil) potentially reduced the oxidative stress and cardiotoxicity.

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Conflicts of interest

There are no conflicts of interest.

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