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Protective effect of caffeic acid on cardiac markers and lipid peroxide metabolism in cardiotoxic rats: an in vivo and in vitro study

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Abstract

Myocardial infarction affects a large population in the world. Lipid peroxide metabolism plays an important role in the pathology of myocardial infarction. This study aims to evaluate the preventive effect of caffeic acid on lipid peroxides, antioxidants, cardiac marker enzymes, and histopathological findings in isoproterenol (ISO)-induced myocardial-infarcted male Wistar rats. Myocardial infarction was induced in rats by subcutaneous injection of ISO (100 mg/kg) at an interval of 24 hours for 2 days. The ISO-induced rats showed significant increase in the levels of thiobarbituric acid reactive substances, lipid hydroperoxides in the heart, plasma uric acid, and serum cardiac marker enzymes, and significant decrease in the activities of heart superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione-*S*-transferase, and the levels of reduced glutathione, vitamin E, and vitamin C in the plasma and heart. Oral pretreatment with caffeic acid (15 mg/kg) daily for 10 days showed significant decrease in the levels of serum cardiac marker enzymes, heart lipid peroxidation products and plasma uric acid and significant increase in the levels of antioxidant system. Histopathology of myocardium also confirmed the protective effect of caffeic acid in myocardial-infarcted rats. In vitro study on total antioxidant activity (2,2'-azinobis-[3-ethylbenzothiazoline-6-sulfonic acid]$^{+}$ assay) confirmed the strong antioxidant action of caffeic acid. Thus, the present study revealed that caffeic acid ameliorates cardiac damage in ISO-induced myocardial infarction by maintaining lipid peroxide metabolism due to its free radical scavenging and antioxidant effects. A diet containing caffeic acid may be beneficial to myocardial infarction.

1. Introduction

Cardiovascular diseases (CVDs) remain the principal cause of death in both developed and developing countries, accounting for roughly 20% of all worldwide deaths per year. Cardiovascular diseases in India cause 3 million deaths per year, accounting for 25% of all mortality. The World Health Organization predicts that deaths due to circulatory system diseases are projected to double between 1985 and 2015 [1].

Myocardial infarction (MI) is the acute condition of necrosis of the myocardium that occurs as a result of imbalance between coronary blood supply and myocardial demand [2]. Isoproterenol (ISO) causes oxidative stress in the myocardium resulting in gross and microscopic infarct in rat heart muscle [3]. Isoproterenol-induced myocardial necrosis also showed membrane permeability alterations, which bring about the loss of function and integrity of myocardial membranes [4]. It has been reported that ISO produces free radicals and stimulates lipid peroxidation, which may be a causative factor for irreversible damage to the myocardial membrane [5]. The induction of MI in experimental animals by ISO is probably due to its action on the sarcolemmal membrane, stimulation of adenylate cyclase, activation of Na^{+} and Ca^{2+} channels, exaggerated Ca^{2+} inflow, and energy consumption leading to cellular death [6]. The pathophysiological changes that take place in the rat's heart after MI induced by ISO administration is comparable to the changes taking place after MI in humans [7]. By studying the biochemical alterations that take place in an animal model, it is possible to gain more insight into the mechanisms leading to the altered metabolic process in human MI.

Dietary factors play a vital role in the development of various human diseases including CVDs. Phenolic compounds form a substantial part of plant foods. Most of these phenolic compounds are antioxidants in vitro [8], and antioxidants may protect against CVDs. Hydroxycinnamic acids are the major classes of phenolic compounds, which

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are found in almost every plant [9,10]. Phenolic acids are hydroxylated derivatives of benzoic and cinnamic acids. The most representative of hydroxycinnamic acids is caffeic acid. Caffeic acid (3,4-dihydroxycinnamic acid) is one of the most common phenolic acids and occurs in fruits [11], grains [12], and dietary supplements [13] for human consumption as simple ester with quinic acid or saccharides. The intake of caffeic acid from foods, mainly from tomatoes and potatoes, was estimated to be about 0.2 mg/kg body weight per day [14]. It has been reported that caffeic acid possesses several pharmacological properties like antioxidant [15], free radical scavenging [16], antimutagenic [17], anticarcinogenic [18,19], lipoxygenase inhibitor [20], and chelator of metal ions [21].

Recently, phenolic acids have received much attention because of their role in the prevention of many human diseases, particularly atherosclerosis and cancer due to their antioxidant properties [11]. Very few reports are available on the effect of nonflavonoids such as phenolic acids and their mechanism in MI. A previous scientific report has shown that caffeic acid inhibits oxidation of low-density lipoprotein in vitro and might therefore protect against CVDs [20]. The mechanism of action of ISO-induced MI is not clearly understood. Biological compounds with antioxidant properties contribute to the protection of cells and tissues against deleterious effects of reactive oxygen species and other free radicals [22]. There are no in vivo studies available on the effect of caffeic acid in MI. Therapeutic intervention that could improve impaired antioxidant defense mechanisms or diminish free radical production in the ischemic myocardium has been of great interest [23]. By decreasing free radical production, one can prevent cardiac tissue damage and protect the heart from free radical–mediated cardiac damage in ISO-induced rats. Recently, there has been an upsurge of interest to explore the cardioprotective potential of natural products [24]. Natural products have lesser adverse effects than synthetic drugs. The mechanism(s) of cardioprotective effect of caffeic acid has not been investigated. Lipid peroxide metabolism plays an important role in the pathogenesis of MI. A detailed study is necessary to know whether the antioxidant activity of caffeic acid plays any role in the lipid peroxide–mediated cardiac damage in MI. In this context, an attempt has been made to elucidate the mechanism of protective effect of caffeic acid in ISO-induced cardiac damage with reference to lipid peroxidation, antioxidants, and cardiac enzymes that play an important role in cardiac damage. In addition to this, in vitro effect of caffeic acid on 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid $(ABTS)^+$ (total antioxidant activity) was studied.

2. Materials and methods

2.1. Experimental animals and diets

All the experiments were performed with male albino Wistar rats (*Rattus norvegicus*) weighing 170 to 200 g obtained from the Central Animal House, Rajah Muthiah Institute of Health Sciences, Annamalai University, Tamil Nadu, India. They were housed in polypropylene cages (47 cm × 34 cm × 20 cm) (4 rats per cage) lined with husk, renewed every 24 hours under a 12:12-hour light-dark cycle at around 22°C and had free access to tap water and food. The rats were fed on a standard pelleted diet (Pranav Agro Industries, 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 Animal Ethical Committee of Annamalai University (approval no. 556:20.3.2008).

2.2. Drug and chemicals

Caffeic acid, isoproterenol hydrochloride, butylated hydroxyl toluene, nitroblue tetrazolium, phenazine methosulfate, glutathione, nicotinamide adenine dinucleotide, and 1-chloro-2,4-dinitrobenzene were purchased from Sigma Chemical, St Louis, MO. All the other chemicals used were of analytical grade.

2.3. Study design

A pilot study was conducted with 3 different doses of caffeic acid (5, 10, and 15 mg/kg) to determine the dose-dependent effect of caffeic acid in ISO-induced myocardial-infarcted rats. It was observed that after 10 days of experiment, caffeic acid pretreatment at the doses of 5, 10, and 15 mg/kg significantly ($P < .05$) lowered the elevated levels of serum creatine kinase (CK) in ISO-induced myocardial-infarcted rats. From the results, it was observed that 15 mg/kg of caffeic acid showed the highest significant effect than the other 2 doses (5 and 10 mg). Hence, we have chosen the highest dose (15 mg/kg) for our study.

In this experiment, a total of 32 rats were used. Eight rats were used in each group. Two rats from each group were used for histological study. The 4 groups were as follows: group I, normal control rats; group II, normal rats treated with caffeic acid (15 mg/kg); group III, rats subcutaneously injected with ISO twice at an interval of 24 hours (on the 11th and 12th day); group IV, rats pretreated with caffeic acid (15 mg/kg) and then subcutaneously injected with ISO twice at an interval of 24 hours (on the 11th and 12th day). Caffeic acid was dissolved in 0.5% dimethyl sulfoxide and administered to rats orally using an intragastric tube daily for a period of 10 days. Normal and ISO control rats received 0.5% dimethyl sulfoxide alone.

At the end of the experimental period after 12 hours of second ISO injection (on the 13th day), all the rats were anesthetized and killed by cervical decapitation. Blood was collected and plasma and serum were separated for various biochemical estimations.

2.4. Processing of heart tissue

The heart tissues were excised immediately from the rats, washed off blood with ice-chilled physiologic saline, and stored in ice-cold containers. A known weight of the heart tissue was homogenized in 5 mL of 0.1 M/L Tris-HCl (pH 7.4) buffer solution. The homogenate was centrifuged and the supernatant was used for the estimation of various biochemical parameters.

2.5. Biochemical estimations

Heart thiobarbituric acid reactive substances (TBARS) were estimated by the method of Fraga et al [25]. The heart tissue homogenate was prepared in Tris-HCI buffer (pH 7.5). One milliliter of the tissue homogenate was treated with 2.0 mL of TBA-TCA-HCI reagent and mixed thoroughly. The mixture was kept in boiling water bath for 15 minutes. After cooling, the tubes were centrifuged for 10 minutes and the supernatant was taken for measurement. The absorbance was read at 535 nm against reagent blank.

Heart lipid hydroperoxides (LOOH) were estimated by the method of Jiang et al [26]. Fox reagent (1.8 mL) was mixed with 0.2 mL of the heart homogenate and incubated for 30 minutes at room temperature. The color developed was read at 560 nm.

Superoxide dismutase (SOD) activity was assayed in the heart by the method of Kakkar et al [27]. Heart tissue homogenate (0.5 mL) was diluted to 1.0 mL with distilled water. Afterwards, 2.5 mL of ethanol and 1.5 mL of chloroform (all reagents chilled) were added. This mixture was shaken and then centrifuged. The enzyme activity in the supernatant was determined. The assay mixture contained 1.2 mL of sodium pyrophosphate buffer, 0.1 mL of phenazine methosulfate, 0.3 mL of nitroblue tetrazolium, 0.2 mL of reduced nicotinamide adenine dinucleotide (NADH), appropriately diluted enzyme preparation, and distilled water in a total volume of 3.0 mL. The reaction was started by the addition of NADH. After incubation at 30°C for 90 seconds, the reaction was arrested by the addition of 1.0 mL of glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4.0 mL of *n*-butanol. The intensity of the chromogen in the butanol layer was measured at 560 nm against butanol blank.

The activity of catalase in the heart was assayed by the method of Sinha [28]. To 0.9 mL of phosphate buffer, 0.1 mL of heart tissue homogenate and 0.4 mL of H_2O_2 were added. After 60 seconds, 2.0 mL of dichromate-acetic acid mixture was added. The tubes were kept in a boiling-water bath for 10 minutes and the color developed was read at 620 nm.

Glutathione peroxidase (GPx) activity in the heart was assayed by the method of Rotruck et al [29]. To 0.2 mL of Tris buffer, 0.2 mL of ethylene diamine tetraacetic acid, 0.1 mL of sodium azide, and 0.5 mL of heart tissue homogenate were added. To the mixture, 0.2 mL of glutathione followed by 0.1 mL of H_2O_2 was also added. The contents were mixed well and incubated at 37°C for 10 minutes along with a tube containing all the reagents except the sample. After 10 minutes, the reaction was arrested by the addition of 0.5 mL of 10% trichloroacetic acid, centrifuged, and the supernatant was used for the estimation of glutathione.

Glutathione reductase (GRx) activity was assayed in heart tissue homogenate by the method of Horn and Burns [30]. To 0.2 mL of phosphate buffer, 0.1 mL of heart homogenate, 0.1 mL of oxidized glutathione, 0.1 mL of flavine adenine dinucleotide, and 0.5 mL of ethylenediaminetetraacetic acid were added. The control tubes were incubated at 37°C for 15 minutes. Afterwards, 0.1 mL of NADH solution was added to all the tubes. The reaction rate was monitored at 340 nm for 5 minutes and the change in absorbance was measured.

Activity of glutathione-*S*-transferase (GST) was measured in the heart by the method of Habig and Jakoby [31]. The reaction mixture containing 1.0 mL of phosphate buffer, 0.1 mL of 1-chloro-2,4-dinitrobenzene, and 0.1 mL of heart homogenate was made up to 3.0 mL with distilled water. The reaction mixture was incubated at 37°C for 5 minutes. Afterwards, 0.1 mL of reduced glutathione (GSH) was added and change in absorbance was measured at 340 nm for 3 minutes at 30-second interval in a Systronics UV-visible spectrophotometer (Hyderabad, India).

Vitamin C in the plasma and heart was estimated by the method of Omaye et al [32]. Plasma/heart tissue homogenate (0.5 mL) was mixed thoroughly with 1.5 mL of 6% trichloroacetic acid and centrifuged for 20 minutes at 3500*g*. To 0.5 mL of the supernatant, 0.5 mL of dinitrophenylhydrazine reagent was added and mixed well. The tubes were allowed to stand at room temperature for 3 hours, removed, and placed in ice-cold water. Afterwards, 2.5 mL of 85% sulfuric acid was added to all the tubes and allowed to stand for 30 minutes at room temperature. The color developed was read at 530 nm.

The level of vitamin E in the plasma and heart was estimated by the method of Baker et al [33]. To 0.5 mL of plasma/heart tissue homogenate, 1.5 mL of ethanol and 2.0 mL of petroleum ether were added, mixed, and centrifuged. The supernatant was evaporated to dryness at 80°C. To this, 0.2 mL of 2,2'-dipyridyl solution and 0.2 mL of ferric chloride were added. Afterwards, all the tubes were mixed well and kept in the dark for 5 minutes and 2.0 mL of *n*-butanol was added. The red color developed was read at 520 nm.

Estimation of GSH in the heart was done by the method of Ellman [34]. A known weight of the heart tissue was homogenized in phosphate buffer. From this, 0.5 mL of tissue homogenate was pipetted out and precipitated with 2.0 mL of 5% trichloroacetic acid. After centrifugation, 1.0 mL of the supernatant was taken. To this, 0.5 mL of Ellman reagent and 3.0 mL of phosphate buffer were added. The yellow color developed was read at 412 nm.

Protein content in the heart homogenate was determined by the method of Lowry et al [35]. Heart tissue homogenate (0.5 mL) was precipitated with 0.5 mL of 10% trichloroacetic acid and centrifuged for 10 minutes and the

Table 1
Effect of caffeic acid on the levels TBARS and LOOH in the heart of normal and ISO-induced myocardial-infarcted rats

Groups	Normal control	Caffeic acid (15 mg/kg)	ISO (100 mg/kg)	Caffeic acid (15 mg/kg) + ISO
Heart TBARS (m mol/100 g wet tissue)	0.65 ± 0.06[a]	0.64 ± 0.06[a]	1.40 ± 0.08[b]	0.80 ± 0.05[c]
Heart LOOH (m mol/100 g wet tissue)	29.00 ± 2.33[a]	28.79 ± 2.30[a]	53.30 ± 5.04[b]	38.16 ± 3.36 [c]

Each value is mean ± SD for 6 rats in each group; values not sharing a common superscript (a, b, c) differ significantly with each other ($P < .05$; Duncan's multiple range test).

precipitate was dissolved in 1.0 mL of 0.1 N sodium hydroxide. From this, 0.1 mL of aliquot was taken and made up to 1.0 mL with distilled water. Afterwards, 4.5 mL of alkaline copper reagent was added and allowed to stand at room temperature for 10 minutes. After incubation, 0.5 mL of Folin-Ciocalteau reagent was added and the blue color developed was read at 620 nm after 20 minutes.

The activities of serum CK-MB (Agappe Diagnostics, Kerala, India) and lactate dehydrogenase (LDH) (Qualigens Diagnostics, Mumbai, India) were assayed using standard commercial kits. The level of plasma uric acid was estimated by a standard reagent kit (Ranboxy Laboratories, United Kingdom).

2.6. Histopathological examination

Heart tissues obtained from all the groups were washed immediately with saline and then fixed in 10% buffered neutral formalin solution. After fixation, the heart tissues were processed by embedding in paraffin. The heart tissues were then sectioned and stained with hematoxylin and eosin and examined under high-power microscope (100×), and photomicrographs were taken.

2.7. Total antioxidant activity assay ($ABTS^+$ assay)

Total antioxidant potential of caffeic acid in vitro was determined by the $ABTS^+$ scavenging assay, as described by Miller et al [36]. The reaction mixture contained $ABTS^+$, caffeic acid (20-100 μM), and buffer in a total volume of 3.5 mL. The absorbance was measured at 734 nm in a Systronics UV-visible spectrophotometer [37,38].

2.8. Statistical analysis

Statistical analysis was performed by 1-way analysis of variance followed by Duncan's multiple range test using Statistical Package for the Social Science software package version 12.00 (SPSS, Chicago, IL). Results were expressed as mean ± SD for 6 rats in each group. P values < .05 were considered significant.

3. Results

Rats induced with ISO showed significant ($P < .05$) increase in the levels of TBARS and LOOH in the heart compared with normal control rats. Oral pretreatment with caffeic acid (15 mg/kg) to ISO-induced rats significantly ($P < .05$) decreased the levels of TBARS and LOOH in the heart compared with ISO-alone–induced rats (Table 1).

Table 2 represents the effect of caffeic acid on the activities of SOD, catalase, GPx, GRx, and GST in the heart of normal and ISO-induced rats. The ISO-induced rats showed a significant ($P < .05$) decrease in the activities of these enzymic antioxidants in the heart compared with the normal control rats. Pretreatment with caffeic acid (15 mg/kg) enhanced the activities of these enzymes significantly ($P < .05$) compared with ISO-alone–induced rats.

Table 3 shows the effect of caffeic acid on the degree of histological changes in myocardial tissues in normal and ISO-induced rats. Normal rats (group 1) and normal rats treated with caffeic acid (15 mg/kg) showed no cardiac muscle separation, broken cardiac fibers, hemorrhage, and mononuclear inflammatory infiltrate in cardiac tissues (A). Group III (ISO-treated rats) showed cardiac muscle separation, broken cardiac fibers, hemorrhage, and mononuclear infiltrate (+++). Pretreatment with caffeic acid (15 mg/kg) showed no cardiac muscle separation, broken cardiac fibers, and hemorrhage (A) with few mononuclear infiltrate (+).

The ISO-induced rats exhibited significantly ($P < .05$) lowered levels of plasma vitamin C and vitamin E and

Table 2
Effect of caffeic acid on the activities of SOD catalase, GPx, GRx, and GST in the heart of normal and ISO-induced myocardial-infarcted rats

Groups	Normal control	Caffeic acid (15 mg/kg)	ISO (100 mg/kg)	Caffeic acid (15 mg/kg) + ISO
SOD (U/mg protein)	14.71 ± 1.21[a]	14.88 ± 1.04[a]	7.40 ± 0.67[b]	12.12 ± 1.13[c]
Catalase (μmol of H_2O_2 consumed/[min mg protein])	8.25 ± 0.06[a]	8.31 ± 0.07[a]	4.41 ± 0.03[b]	7.40 ± 0.06[c]
GPx (μg of GSH consumed/[min mg protein])	6.94 ± 0.52[a]	6.99 ± 0.52[a]	2.61 ± 0.14[b]	5.51 ± 0.41[c]
GRx (nmol of NADPH oxidized/[min 100 mg protein])	9.44 ± 0.87[a]	9.65 ± 0.71[a]	5.47 ± 0.48[b]	8.22 ± 0.76[c]
GST (nmol of CDNB conjugated/[min mg protein])	800.61 ± 70.49[a]	801.62 ± 78.61[a]	514.71 ± 45.28[b]	721.66 ± 67.53[c]

Each value is mean ± SD for 6 rats in each group; values not sharing a common superscript (a, b, c) differ significantly with each other ($P < .05$; Duncan's multiple range test). SOD unit: 1 unit is defined as the enzyme concentration required to inhibit the OD at 560 nm of chromogen production by 50% in 1 minute. NADPH indicates reduced nictotinamide adenine dinucelotide phosphate; CDNB, 1-chloro-2,4-dinitrobenzene.

Table 3
Effect of caffeic acid on the degree of histologic changes in myocardial tissues in normal and ISO-induced myocardial-infarcted rats

Groups	Normal	Caffeic acid (15 mg/kg)	ISO (100 mg/kg)	Caffeic acid (15 mg/kg) + ISO
Cardiac muscle separation	A	A	+++	A
Broken cardiac fibers	A	A	+++	A
Hemorrhage	A	A	+++	A
Mononuclear inflammatory infiltrate in cardiac tissue	A	A	+++	+

Photomicrographs were used to evaluate the damage in the heart tissues: A, no changes; +, mild changes; +++, marked changes.

significantly ($P < .05$) increased levels of plasma uric acid compared with normal control rats. Pretreatment with caffeic acid (15 mg/kg) significantly ($P < .05$) increased the levels of vitamin E and vitamin C and significantly ($P < .05$) lowered the levels of plasma uric acid compared with ISO-alone–induced rats (Fig. 1).

The ISO-induced rats showed significantly ($P < .05$) decreased levels of vitamin C, vitamin E, and GSH in the heart compared with normal control rats. Prior treatment with caffeic acid (15 mg/kg) to ISO-induced rats significantly ($P < .05$) increased the levels of heart vitamin C, vitamin E, and GSH compared with ISO alone induced rats (Figs. 2 and 3).

Fig. 4 represents the effect of caffeic acid on the activities of serum CK-MB and LDH in normal and ISO-induced rats. Rats induced with ISO showed a significant ($P < .05$) increase in the activities of these enzymes in the serum ($P < .05$) compared with normal control rats. Pretreatment with caffeic acid (15 mg/kg) significantly ($P < .05$) decreased the activities of these enzymes in ISO-induced rats compared with ISO-alone–induced rats.

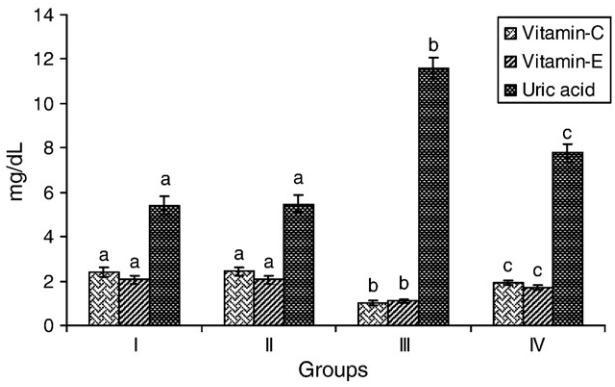

Fig. 1. Effect of caffeic acid on the levels of plasma vitamin C, vitamin E, and uric acid in the normal and ISO-induced myocardial-infarcted rats. Group I, normal control; group II, normal + caffeic acid (15 mg/kg); group III, ISO control (100 mg/kg); group IV, caffeic acid (15 mg/kg) + ISO. Each column is mean ± SD for 6 rats in each group. Columns that have different letters (a, b, c) differ significantly with each other ($P < .05$; Duncan's multiple range test).

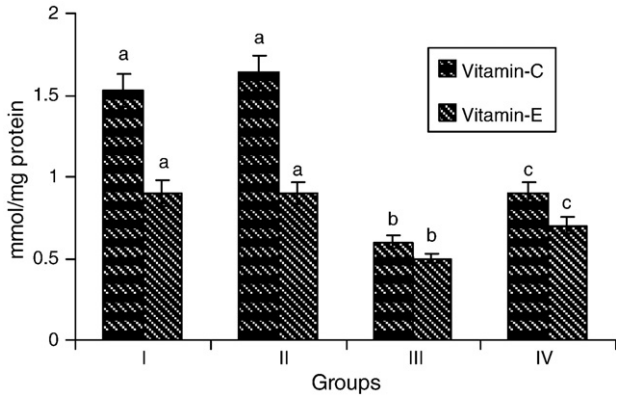

Fig. 2. Effect of caffeic acid on the levels of heart vitamin C and vitamin E in the normal and ISO-induced myocardial-infarcted rats. Group I, normal control; group II, normal + caffeic acid (15 mg/kg); group III, ISO control (100 mg/kg); group IV, caffeic acid (15 mg/kg) + ISO. Each column is mean ± SD for 6 rats in each group. Columns that have different letters (a, b, c) differ significantly with each other ($P < .05$; Duncan's multiple range test).

For all the biochemical parameters studied, treatment with caffeic acid (15 mg/kg) daily for a period of 10 days to normal control rats did not show any significant effect.

Histopathological changes in the heart tissue of all groups were evaluated microscopically (Fig. 5A-D). Normal rat's heart showed normal cardiac fibers (group I; Fig. 5A), and caffeic acid (15 mg/kg)–treated heart tissue showed normal cardiac fibers without any pathological changes (group II; Fig. 5B). However, ISO-induced rat's heart showed cardiac damage and mononuclear inflammatory infiltrate in the endocardium and myocardium (group III; Fig. 5C). The ISO-induced heart also showed cardiac muscle fiber separation and broken fibers with hemorrhage (group III; Fig. 5C1). Caffeic acid (15 mg/kg)–pretreated ISO-induced rat's heart showed reduced cardiac damage with few inflammatory cells (group IV; Fig. 5D).

Fig. 6 shows the percentage scavenging effect of caffeic acid on ABTS radical (total antioxidant activity). Caffeic

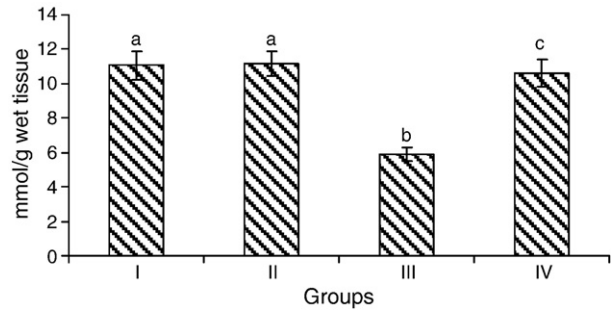

Fig. 3. Effect of caffeic acid on the levels of heart GSH in the normal and ISO-induced myocardial-infarcted rats. Group I, normal control; group II, normal + caffeic acid (15 mg/kg); group III, ISO control (100 mg/kg); group IV, caffeic acid (15 mg/kg) +ISO. Each column is mean ± SD for 6 rats in each group. Columns that have different letters (a, b, c) differ significantly with each other ($P < .05$; Duncan's multiple range test).

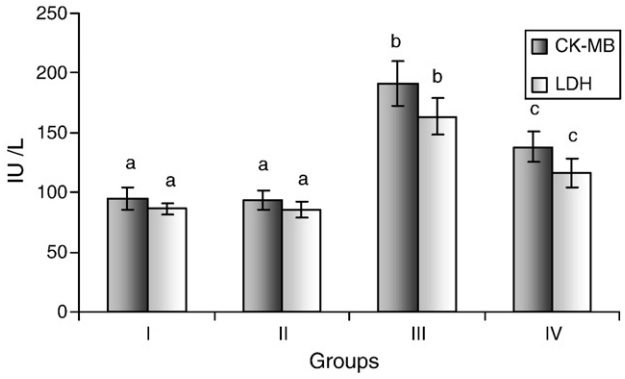

Fig. 4. Effect of caffeic acid on the activities of serum CK-MB and LDH in the normal and ISO-induced myocardial-infarcted rats. Group I, normal control; group II, normal + caffeic acid (15 mg/kg); group III, ISO control (100 mg/kg); group IV, caffeic acid (15 mg/kg) + ISO. Each column is mean ± SD for 6 rats in each group. Columns that have different letters (a, b, c) differ significantly with each other ($P < .05$; Duncan's multiple range test).

acid scavenges ABTS radicals in vitro in a dose-dependent manner (20, 40, 60, 80, and 100 μM). The percentage scavenging effect of caffeic acid increases with increasing concentration. The percentages scavenging of caffeic acid on $ABTS^+$ at the concentrations of 20, 40, 60, 80, and 100 μM of caffeic acid were 16.37, 32.74, 49.11, 65.48, and 81.85, respectively. The percentage scavenging of caffeic acid on ABTS radical at the concentration of 100 μmol was found to be 81.85%.

4. Discussion

In the present study, pretreatment with caffeic acid exhibited significant protection against ISO-induced biochemical and histopathologic changes and reduced cardiac damage in ISO-induced rats. The cardioprotective mechanism(s) appears to be through modulation of lipid peroxides and various antioxidant parameters, thereby improving the overall antioxidant defense of the myocardial tissue. In vitro study of caffeic acid also confirmed the antioxidant effect of caffeic acid.

Development of therapies to prevent the generation of free radicals could influence the progression of oxidative damage induced by ISO. Oxidative stress is defined as a shift in the normal prooxidant/antioxidant balance due to formation of reactive oxygen species [39]. Isoproterenol administration produced oxidative stress–induced lipid peroxidation in heart tissue. Increased lipid peroxidation may be due to the oxidation of ISO to semiquinone, which reacts with oxygen to produce superoxide anions (O^-_2) and H_2O_2. [22,40]. Catecholamines readily form chelate complexes with metal ions such as iron, copper, and manganese, which strongly catalyze oxidation of catecholamines [40]. Another report showed that copper and iron are mobilized in the coronary flow after myocardial ischemia [41]. Both these ions are present in the coronary flow fraction in a redox active form that supports free radical–mediated deleterious reactions [41]. Thus, it is clear that oxidation of ISO involved reactive oxygen species, which may cause peroxidative damage to cardiac tissue.

Lipid peroxide metabolism plays an important role in the pathogenesis of MI. A significant increase in the levels of lipid peroxidation products such as TBARS and LOOH in the heart clearly indicates increased oxidative stress in ISO-induced rats. Alterations in the metabolism of lipid peroxides are closely associated with myocardial damage due to free radicals produced by ISO. Prior treatment with caffeic acid decreased the levels of lipid peroxidation products in ISO-induced rats. Thus, caffeic acid scavenges the lipid peroxidation products produced excessively by ISO protected the cardiac tissue because of its antilipid peroxidation effect. In this context, previous studies also reported the scavenging action of caffeic acid on hydroxyl, superoxide, and lipid radicals [42-44].

Reactive oxygen species are generated from the leakage of electrons into oxygen from various systems. In our body, endogenous antioxidant enzymatic defense is a very important source to neutralize the oxygen free radical–mediated tissue injury [45]. Free radical scavenging enzymes such as catalase, SOD, and GPx are the first line of cellular defense against oxidative injury, decomposing O_2 and H_2O_2 before their interaction to form the more reactive hydroxyl radical. The equilibrium between these enzymes is an important process for the effective removal of oxygen stress in intracellular organelles. In ISO-induced rats, the activities of heart tissue antioxidant enzymes were decreased. During MI, SOD and catalase are structurally and functionally impaired by free radicals resulting in myocardial damage. The decrease in SOD and catalase may be due to the involvement of superoxide and hydrogen peroxide free radicals in myocardial cell damage mediated by ISO [46]. Prior treatment with caffeic acid improved the activities of SOD and catalase by scavenging superoxide and hydrogen peroxides produced by ISO.

Normal activities of GSH-dependent enzymes such as GPx, GRx, and GST are essential for maintaining the antioxidant status. The decreased activity of these enzymes in the heart is due to increased lipid peroxidation in ISO-induced rats. Increased lipid peroxidation resulted in decreased levels of GSH. Thus, the decreased levels of GSH resulted in decreased activities of GPx, GRx, and GST in ISO-induced rats. Increasing GSH content can prevent cellular damage. Thus, the observed increased content of GSH prevented cellular damage and enhanced the activities of GSH-dependent enzymes in caffeic acid–pretreated ISO-induced rats. The effect of caffeic acid on GSH-dependent enzymes and GSH clearly indicates the antioxidant nature of caffeic acid.

The second line of defense consists of the nonenzymatic scavengers, namely, ascorbic acid, α-tocopherol, ceruloplas-

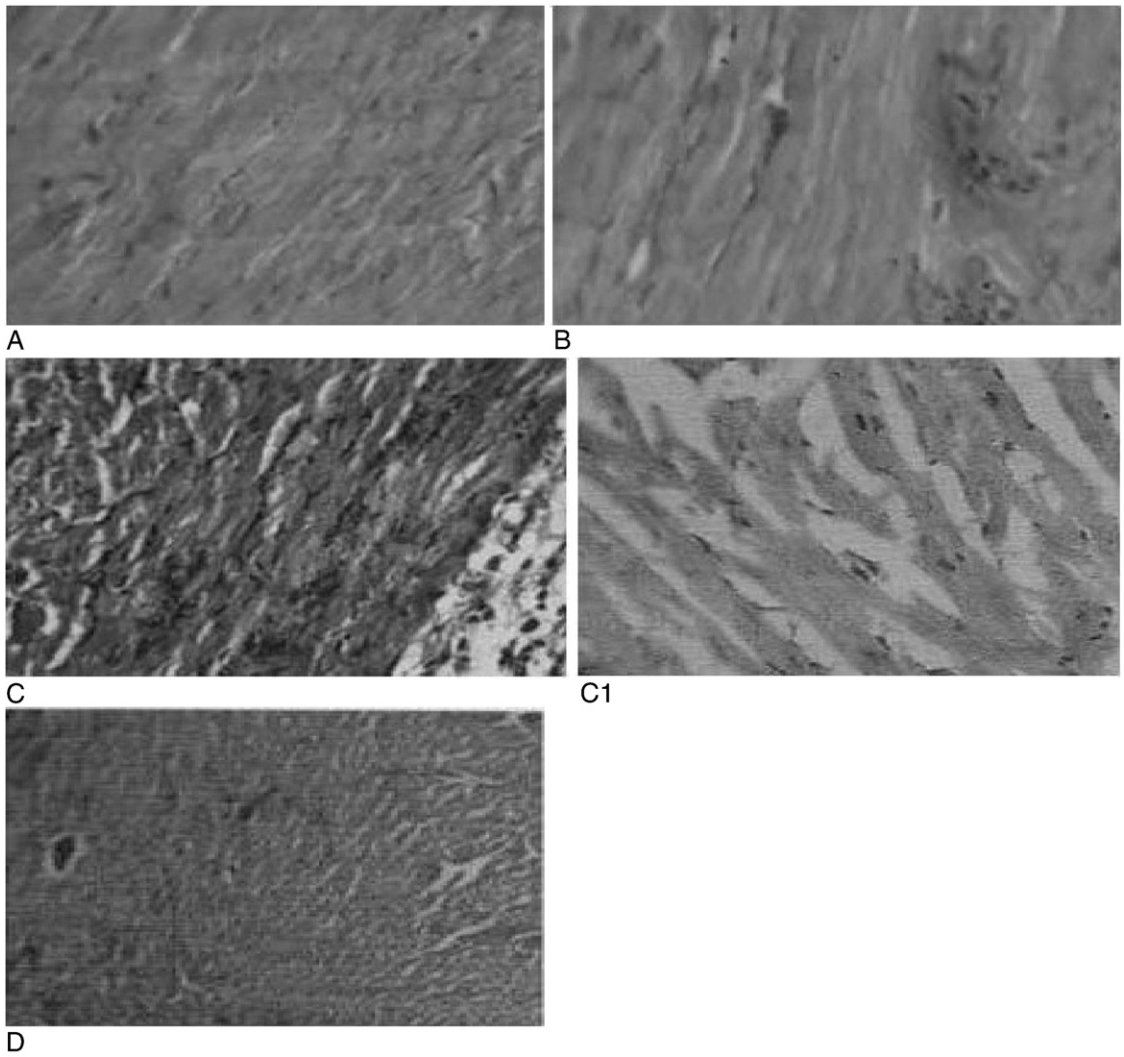

Fig. 5. A-D, Effect of caffeic acid on the histopathology of the myocardium. A, Normal rat's heart (group I) showing normal cardiac fibers (hematoxylin and eosin, 100×). B, Caffeic acid (15 mg/kg)–treated rat's heart tissue (group II) showing normal cardiac fibers without any changes (hematoxylin and eosin, 100×). C, Isoproterenol-induced rat's heart (group III) showing cardiac damage and mononuclear inflammatory infiltrate in the endocardium and myocardium (hematoxylin and eosin, 100×). C1, Isoproterenol-induced rat's heart (group III) showing cardiac muscle separation, broken fibers, and hemorrhage (hematoxylin and eosin, 100×). D, Caffeic acid (15 mg/kg) + ISO-induced rat's heart (group IV) with reduced damage and few inflammatory cells (hematoxylin and eosin, 100×).

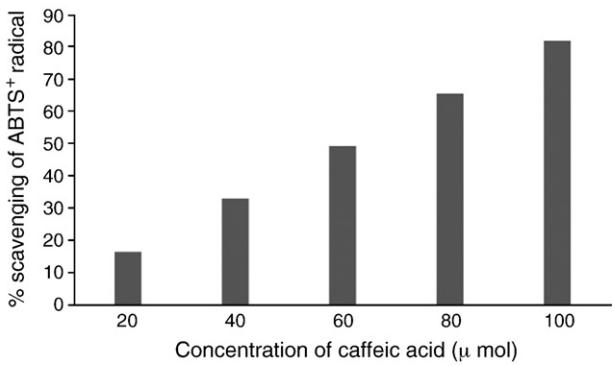

Fig. 6. In vitro scavenging effect of caffeic acid on ABTS radical (total antioxidant activity).

min, and sulfhydryl-containing compounds, which scavenge the residual free radicals escaping from decomposition by the antioxidant enzymes. It has been reported that an inverse correlation exists between plasma levels of vitamin E and mortality from ischemic heart disease [47]. Ascorbic acid present in aqueous environment has multiple antioxidant properties including the ability to regenerate α-tocopheryl radicals present on the surface of the membrane [48]. The lowered concentrations of vitamins C and E observed in ISO-induced rats might be due to neutralizing increased production of free radicals. Prior treatment with caffeic acid enhanced the levels of these vitamins in ISO-induced rats. The increased concentration of these vitamins may protect the heart against ISO-mediated free radicals in caffeic acid–pretreated ISO-induced group.

Serum uric acid is considered to be a risk factor for MI [49]. Increased levels of serum uric acid indicate increased production of free radicals in MI. Prior treatment with caffeic acid significantly reduced serum uric acid levels compared with ISO-induced rats. The decreased levels of lipid peroxidation observed in caffeic acid–pretreated ISO-induced rats resulted in decreased levels of uric acid. This effect shows the antioxidant potential of caffeic acid.

The diagnostic marker enzymes of MI such as CK-MB and LDH were increased in the serum of ISO-induced myocardial-infarcted rats. The increased level of these enzymes is an indication of the severity of ISO-induced necrotic damage to the myocardial membrane. Assessment of the magnitude and persistence of elevation of CK-MB activity in the plasma is the best way to estimate the extent of infarction. Creatine kinase–MB and LDH being myocardial enzymes, they are leaked out from the cardiac tissue to blood upon the development of degenerative changes in myocardial cell membranes. Caffeic acid pretreatment decreased the activities of these serum marker enzymes in ISO-induced rats. Thus, caffeic acid prevents the leakage of these marker enzymes from the heart into blood by reducing cardiac tissue damage by its antioxidant as well as cardioprotective effects.

In vivo experiments carried out in the present study demonstrates inhibition of lipid peroxidation, enhancement of SOD and catalase activities, improvement in GSH levels and activities of GSH-dependent enzymes, enhancement of vitamins E and C levels, and reduction of uric acid levels and activities of CK-MB and LDH in caffeic acid–pretreated groups in ISO-induced rats, which further implies that the cardioprotective effect of caffeic acid may be by virtue of its free radical scavenging and antioxidant effects by maintaining lipid peroxide metabolism. Histopathology of caffeic acid–pretreated ISO-induced heart showed reduced cardiac damage. Thus, histopathological findings of the present study confirmed the biochemical observations of this study. Furthermore, caffeic acid is a phenyl propanoid. According to the chemical structure, caffeic acid contains 2 hydroxyl groups on the benzene moiety. These 2 hydroxyl groups also enhance the free radical scavenging effect of caffeic acid.

To know the antioxidant potential of caffeic acid in vitro, we studied $ABTS^+$ scavenging (total antioxidant activity) effect of caffeic acid in different concentrations. Generation of the $ABTS^+$ radical cation forms the basis of one of the spectrophotometric methods that have been applied to the total antioxidant activities of solutions of pure substances. In this study, caffeic acid scavenges $ABTS^+$ dose dependently. The highest percentage of scavenging effect of caffeic acid on $ABTS^+$ at the concentration of 100 μmol was found to be 81.85%. Thus, caffeic acid has potent antioxidant activity. In vitro study also confirmed the antioxidant effect of caffeic acid. Thus, the free radicals such as superoxide and hydroxyl radicals produced by ISO are effectively scavenged by the antioxidant effect of caffeic acid. The decreased levels of these free radicals resulted in reduced cardiac damage in ISO-induced rats.

According to our findings, a 70-kg person requires 1050 mg of caffeic acid per day. Caffeic acid (15 mg/kg) administration to normal control rats did not show any significant effect in all the biochemical parameters and histology of myocardium studied. This shows the nontoxic nature of caffeic acid up to 15 mg/kg.

In conclusion, caffeic acid, a nonflavonoid, protected the myocardium against ISO-induced cardiac damage by maintaining lipid peroxide metabolism, scavenging free radicals, and improving antioxidant system by its antioxidant effect. The results of this study show that caffeic acid is an effective and safe antioxidant in animal models. A diet containing caffeic acid may be beneficial to MI. Further studies are necessary to promote the use of this drug in the future for MI. The results of the present investigation may trigger a renewed interest in the use of caffeic acid for MI.

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