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Cardioprotective properties of *Crataegus oxycantha* extract against ischemia-reperfusion injury

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ABSTRACT

The aim of the study was to investigate the cardioprotective effect and mechanism of *Crataegus oxycantha* (COC) extract, a well-known natural antioxidant-based cardiotonic, against ischemia/reperfusion (I/R) injury. Electron paramagnetic resonance studies showed that COC extract was capable of scavenging superoxide, hydroxyl, and peroxyl radicals, *in vitro*. The cardioprotective efficacy of the extract was studied in a crystalloid perfused heart model of I/R injury. Hearts were subjected to 30 min of global ischemia followed by 45 min of reperfusion. During reperfusion, COC extract was infused at a dose rate of 1 mg/ml/min for 10 min. Hearts treated with COC extract showed a significant recovery in cardiac contractile function, reduction in infarct size, and decrease in creatine kinase and lactate dehydrogenase activities. The expressions of xanthine oxidase and NADPH oxidase were significantly reduced in the treated group. A significant upregulation of the anti-apoptotic proteins Bcl-2 and Hsp70 with simultaneous downregulation of the pro-apoptotic proteins cytochrome *c* and cleaved caspase-3 was observed. The molecular signaling cascade including phospho-Akt (ser-473) and HIF-1α that lead to the activation or suppression of apoptotic pathway also showed a significant protective role in the treatment group. No significant change in phospho-p38 levels was observed. The results suggested that the COC extract may reduce the oxidative stress in the reperfused myocardium, and play a significant role in the inhibition of apoptotic pathways leading to cardioprotection.

Introduction

Ischemia/reperfusion (I/R)-mediated acute myocardial infarction (AMI) is the leading cause of death in the world. The high mortality is due to poor recovery of hearts from AMI and cardiac remodeling induced by progressive necrotic and apoptotic cells in the myocardium (Fliss and Gattinger, 1996; Gottlieb et al., 1994). I/R-induced injury is known to increase the levels of reactive oxygen species (ROS) several-fold which can lead to apoptosis (Jacobson, 1996; Ryter et al., 2007). Various reports suggest that antioxidant therapy after I/R would help the myocardium to recover from ROS-induced damage (Chen et al., 1996; Du et al., 2007). The deleterious effects of ROS on cardiac tissue can be blocked by antioxidant enzymes such as superoxide dismutase and catalase (Ambrosio et al., 1987; Ambrosio et al., 1993; Maulik et al., 1996; Zweier et al., 1989, 1987). These studies indicated that antioxidants capable of scavenging ROS, including reactive oxygen free radicals such as superoxide, hydroxyl, and peroxyl radicals, could have therapeutic advantages to treat I/R-mediated cardiac injury. Thus there is a need to understand and identify suitable antioxidant interventions to salvage the myocardium from I/R-mediated tissue damage and dysfunction.

The ethanolic extract of *Crataegus oxycantha* (COC) is traditionally used as a cardiotonic in China, India, and many European countries. COC contains oligomeric proanthocyanidins (OPC), flavonoids, and polyphenols which are well-known for their antioxidant properties (Rice-Evans et al., 1996; Svedstrom et al., 2002). Several studies have shown that COC extract is effective in quenching ROS, particularly free radicals (al Makdessi et al., 1999; Jayalakshmi et al., 2006; Long et al., 2006). Human subjects treated with COC extract after myocardial infarction have shown improvements in heart rate, reduction in blood pressure, and an

Abbreviations: COC, *Crataegus oxycantha*; I/R, Ischemia/reperfusion; OPC, Oligomeric proanthocyanidin; ROS, Reactive oxygen species; LVDP, Left ventricular developed pressure; CF, Coronary flow; HR, Heart rate; RPP, Rate pressure product; LDH, Lactate dehydrogenase; CK, Creatine kinase; TTC, Triphenyltetrazolium chloride

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increase in the left-ventricular ejection volume (Degenring et al., 2003; Walker et al., 2002). Moreover, meta analysis of a randomized trial with COC extract showed its beneficial role as an adjunctive treatment for chronic heart failure (Pittler et al., 2003). It has also been shown that an alcoholic extract of COC promoted improvement in TCA cycle enzyme activity and protected the mitochondria against isoproteronol-induced cardiac injury (Jayalakshmi and Devaraj, 2004; Jayalakshmi et al., 2006).

Although COC extract has been shown to improve cardiac function, the exact mechanism of action has not yet been elucidated. In the present study, we have investigated the antioxidant property of an ethanolic extract of COC using EPR spectroscopy and cardioprotection in an isolated rat heart model. We have investigated the molecular mechanism and signaling pathways involved in the treatment of I/R-induced myocardial injury with COC extract. We have shown that treatment with COC ethanolic extract improves the cardiac function at both the functional and molecular levels.

Materials and methods

COC seeds were kindly provided by the SBL Private Limited (Delhi, India). 5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide (DEPMPO) was purchased from Dojindo (Kumamoto, Japan). Triphenyltetrazolium chloride (TTC) was purchased from Sigma Diagnostics (St. Louis, MO). Primary antibodies for gp91, xanthine oxidase, HIF-1α, Hsp70 and appropriate secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Primary antibodies for phospho-Akt, Akt, phospho-p38, Bcl-2, cytochrome *c*, and cleaved caspase-3 were purchased from Cell Signaling (Beverly, MA). All other reagents and chemicals used were of analytical grade.

Isolation of OPCs from COC seeds

The OPCs were isolated from COC seeds by the published method (Svedstrom et al., 2002), but with slight modifications. A 1 g amount of powdered seeds was extracted with a mixture of methanol (pH 6.5) and water (7:3), first with 20 ml and then with 15 ml, in an ultrasonic bath for 15 min, after which the mixture was centrifuged at 3000 rpm for 10 min. The sediment was then extracted with 10 ml of methanol in an ultrabath for 15 min, and after centrifugation, the sediment was finally washed with 5 ml of methanol. The resulting supernatants were filtered and extracted to eliminate chlorophyll and lipophilic compounds with light petroleum (3 × 30 ml). The extract was evaporated to dryness and dissolved in 1 ml of methanol–water (1:1). This was adsorbed on the top of a polyamide column used for column chromatography. The mixture was poured into a chromatography column. The column was first eluted with methanol (pH 6.5) (2 × 7 ml) to give polyamide fractions **1** and **2**, and then with 20 ml of acetone–water (7:3) to give fraction **3**. Fraction **2** was evaporated to dryness, dissolved in 0.5 ml of ethanol and adsorbed on a Sephadex LH-20 column. The column was eluted with ethanol, first with 10 ml and then with 15 ml, and two Sephadex fractions, **1** and **2**, were collected. Sephadex fractions **2** and polyamide fraction **3** were combined and the solvents were evaporated under reduced pressure. The residue was dissolved in 1.0 ml of methanol and filtered (Spartan 13, 0.45 mm, Schleicher & Schuell, Dassel, Germany), and 50 µl of sample solution was analyzed by HPLC. OPC standards catechin, epicatechin, epicatechin gallate and catechin gallate were dissolved in 1 ml of methanol and 50 µl of sample was analyzed by HPLC with similar condition adopted for extracting COC.

High-performance liquid chromatography analysis

The HPLC system consisted of a Shimadzu Multisolvent Delivery System, autosampler programmable photodiode array detection (DAD) system 991 (Shimadzu Corporation, Japan) coupled to a dedicated computer. Solvent A was 2.5% aqueous acetic acid, and solvent B was acetonitrile - 2.5% aqueous acetic acid (80:20 v/v). The linear gradients were: solvent B 7–20% in 50 min, 20–40% B from 50 to 60 min, 100% B from 60 to 65 min, followed by washing and reconditioning the column. The flow-rate was 1 ml/min, detection at 279 nm, column: LiChroCart, 250-4, Hypersil ODS (5 mm) (Waters).

Preparation of COC extract for heart experiments

Ethanolic extract of COC was prepared by the reported method (Long et al., 2006). Briefly, dried berries were crushed and extracted using 50% (v/v) aqueous ethanol for 48 h at 4 °C. After vacuum filtration, the extract was evaporated under vacuum to yield an oily residue. The residue was resuspended and dissolved in phosphate-buffered saline (PBS), to give a final concentration of 100 mg/ml at pH 7.4. The aliquots were stored at -20 °C and used throughout the study to maintain consistency.

Evaluation of radical-scavenging ability of COC extract using EPR spectroscopy

The superoxide, hydroxyl, and peroxyl radical-scavenging capability of the COC extract was evaluated using EPR spectroscopy. A mixture of xanthine (0.2 mM) and xanthine oxidase (0.02 U/ml) was used to generate superoxide radicals. Hydroxyl radicals were generated by reacting ferrous ammonium sulphate (0.1 mM) with hydrogen peroxide (0.1 mM). Thermolytic fission of an air-saturated aqueous solution of 2,2'-azobis-2-amidinopropane dihydrochloride (AAPH, 25 mM) at 37 °C was used to generate peroxyl radicals. The EPR measurements were performed in PBS (pH 7.4) containing diethylenetriaminepentaacetate (DTPA, 0.1 mM) and DEPMPO (1 mM) in the presence or absence of 500 µg/ml of COC extract. The superoxide, hydroxyl, and peroxyl radicals were detected as DEPMPO-OOH, DEPMPO-OH and DEPMPO-OOR adducts, respectively, by EPR spectroscopy. The following acquisition parameters were used: modulation amplitude, 1.0 G; receiver time constant, 82 msec; acquisition time, 300 sec (10 × 30 scans); microwave power, 20 mW; microwave frequency, 9.79 GHz; and modulation frequency, 100 kHz. The EPR spectra of the radical adducts were quantified by double-integration.

Isolated heart preparation

Sprague-Dawley rats (body weight 300 - 350 g) were euthanized by intraperitonial injection of 60 mg/kg sodium pentobarbital and heparin (500 IU/kg). After a midline sternotomy, the hearts were rapidly excised and perfused retrogradely at a constant perfusion pressure of 80 mmHg with a modified Krebs' solution containing NaCl (120 mM), $NaHCO_3$ (25 mM), $MgSO_4$ (1.2 mM), KH_2PO_4 (1.2 mM), $CaCl_2$ (1.2 mM), and glucose (11 mM). The perfusate buffer was saturated with a 95% O_2 and 5% CO_2 gas mixture at 37 °C. A latex balloon was inserted into the left ventricle via the left atrium and inflated with 0.4 ml of distilled water, sufficient to produce an end-diastolic pressure of 8 to 12 mmHg. The contractile and hemodynamic functions of the heart were continuously monitored with a computer-based data acquisition system (PC PowerLab with Chart 5 software, ADI Instruments, Colorado Springs, CO). The following data were

collected: coronary flow (CF), left ventricular developed pressure (LVDP), and the rate-pressure-product (RPP), calculated as LVDP × HR (heart rate). The coronary flow rate was measured using a flowmeter with an in-line probe (Transonic Systems, Ithaca, NY).

I/R experimental protocol

Isolated rat hearts were perfused with a modified Krebs buffer solution for 15 min to stabilize the cardiac functions and then subjected to 30 min of global ischemia, followed by 45 min of reperfusion. The hearts were randomly divided into two groups of at least six hearts per group: (i) control (I/R) group, received no treatment; (ii) COC group, received COC extract (1 mg/ml/min) infused through a side-arm for 10 min during the onset of reperfusion. The coronary effluent was collected for the determination of creatine kinase (CK) and lactate dehydrogenase (LDH) activities immediately before ischemia and after 15 min of reperfusion. Myocardial tissue was collected at the end of reperfusion. The tissue was quickly frozen in liquid nitrogen and stored at -80 °C until Western blot could be performed.

LDH and CK assays

Myocardial tissue damage was assessed by determining the level of activities of LDH and CK in the coronary effluent collected before ischemia and after 15 min of reperfusion, using a commercially available kit (Sigma Diagnostics, St. Louis, MO for LDH and Catachem, Bridgeport, CT for CK). The rate of change in absorbance was determined by measurement on a Varian Cary 50 spectrophotometer at 340 nm for 5 min at 25 °C.

Evaluation of myocardial infarct size

The myocardial infarct size was measured in control (I/R) and COC groups, using triphenyltetrazolium chloride (TTC) staining (Walker et al., 1993). TTC stains all living tissue brick-red, leaving the infarct area unstained (white). Initially, the hearts were subjected to 30 min of ischemia followed by 2 h of reperfusion. The reperfusion time was extended to 2 h instead of 45 min in the I/R experimental protocol for TTC staining, to delineate the infarct region clearly. Heart tissues were frozen by storing at -20 °C for 30 min, and then sliced perpendicularly along the long axis from apex to base in 2-mm thick sections. The sections were then incubated for 20 min at 37 °C with 1% TTC in PBS (pH 7.4) and fixed in 10% formalin for 60 min and digitally imaged using a Nikon microscope (Nikon Corporation, Tokyo, Japan). The areas of infarct size (TTC-negative) and at-risk (TTC-positive) were determined using MetaMorph software (Molecular Devices, Sunnyvale, CA). The infarct size was expressed as a percentage of left ventricular area at risk.

Western blot analysis

Heart tissues were homogenized in TN1 lysis buffer containing 50 mM Tris (pH 8.0), 10 mM EDTA, 10 mM $Na_4P_2O_7$, 10 mM NaF, 1% Triton X-100, 125 mM NaCl, 10 mM Na_3VO_4, and 1 μg/ml each of aprotinin and leupeptin. Aliquots of 50 μg of protein from each sample were boiled in sodium dodecylsulfate buffer containing 60 mM Tris (pH 6.8), 2.3% SDS, 10% glycerol, 0.01% bromophenol blue, and 1% 2-mercaptoethanol for 5 min. The protein was separated by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes, and probed with antibodies for gp91, NADPH oxidase, cytochrome *c*, Bcl-2, Hsp70, cleaved caspase-3, phospho-Akt (ser-473), Akt, HIF-1α, and phospho-p38. The primary antibodies were exposed for overnight at 4 °C. Next, horseradish peroxidase-conjugated secondary antibodies were added, and incubated for 1 h at 37 °C. The membranes were then developed by enhanced chemiluminescence. The same membranes were reprobed with antibody for actin. The enhanced chemiluminescence signal was quantified using a scanner and a densitometry program (Scion Image). To quantify the phospho-specific signal in the activated samples, the background was subtracted and then the band was normalized to the amount of actin or total target protein in the lysate (Selvendiran et al., 2007).

Data analysis

The statistical significance of the results was evaluated using one-way ANOVA and a Student t-test analysis. All values were expressed as mean ± SD. Consideration for significance was set at $p < 0.05$.

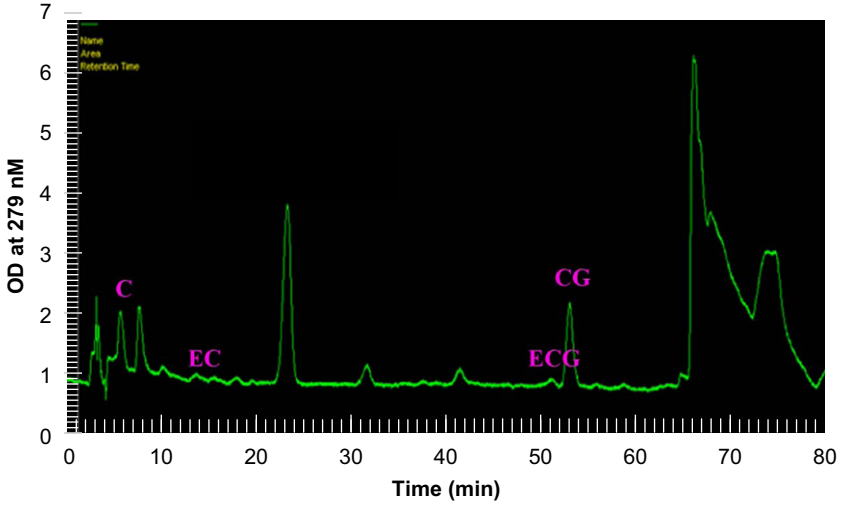

Fig. 1. HPLC profile of a methanolic extract prepared from COC seeds. Quantitative determinations of OPCs were performed using commercially available OPCs as standards. The compositions of catechin (C), epicatechin (EC), and catechin gallate (CG) in the dry powder of COC seeds were 4.52%, 0.6%, 0.6%, respectively. ECG was only present in traces.

Results

Compositions of COC extract

HPLC was used to identify the OPCs including catechin, epicatechin, and catechin galate in the COC extract. The HPLC profile of a methanolic extract prepared from COC seeds is presented in Fig. 1. Quantitative determinations of OPCs were performed using commercially available OPCs as standards. The compositions of catechin, epicatechin, and catechin gallate in the dry powder of COC seeds were 4.52%, 0.6%, 0.6%, respectively.

COC extract scavenges superoxide, hydroxyl and peroxyl radicals

We used spin-trapping EPR spectroscopy and DEPMPO (1 mM) for direct detection of exogenously generated superoxide, hydroxyl and peroxyl radicals. Fig. 2 shows the scavenging effect of the COC extract against superoxide, hydroxyl and peroxyl radicals. COC extract used at a concentration of 500 μg/ml, decreased the superoxide spectrum intensity by more than 50%. Similarly, the hydroxyl radical signal, was inhibited by more than 25% in the presence of 500 μg/ml of COC extract. More importantly, COC extract decreased the peroxyl radicals by more than 50% by 500 μg/ml of COC extract. The EPR results clearly demonstrated that the COC extract was capable of scavenging reactive oxygen radicals.

COC extract improves contractile functions in the reperfused heart

The CF and LVDP were continuously monitored during the perfusion of modified Krebs buffer in COC extract-treated heart and I/R heart. From the LVDP and CF data, the rate pressure product (RPP) was calculated. The data were expressed as a percentage of their pre-ischemic baseline values (Fig. 3). COC-treated hearts showed a significant recovery ($p < 0.05$) in LVDP, CF and RPP compared to untreated I/R group.

COC extract decreases LDH and CK activity in the reperfused heart

The activities of CK and LDH in the heart effluent were used as markers of myocardial injury. Upon treatment with COC extract, the activity of CK was significantly reduced ($p < 0.001$) when compared to untreated I/R groups (Fig. 4A). Fig. 4B shows the levels of LDH activity in the reperfused heart. The COC-treated

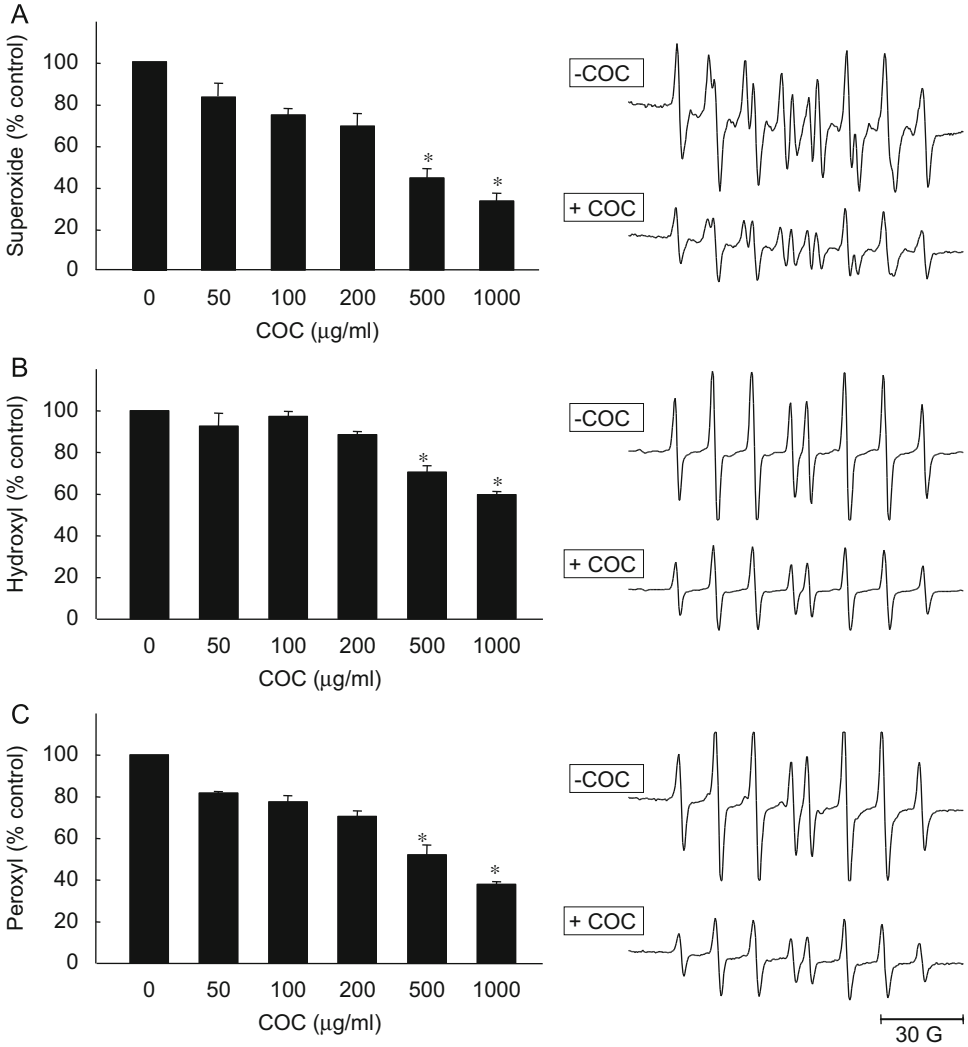

Fig. 2. Scavenging of reactive oxygen free radicals by COC extract. The free radicals were generated as described in the Material and Methods section and measured by EPR spectroscopy using DEPMPO. The EPR spectra (right panels) and quantification (left panels) show a dose-dependent loss of EPR signal intensity corresponding to (A) superoxide, (B) hydroxyl, and (C) peroxyl radicals. $^{*}p < 0.05$ when compared control (-COC).

group showed a significant decrease ($p < 0.001$) in LDH activity compare to untreated I/R hearts.

COC extract attenuates myocardial infarction

Fig. 5 shows representative TTC-stained images as well as infarct areas expressed as a percentage of the left ventricular area in untreated I/R and COC extract-treated I/R hearts obtained after 2 h of reperfusion. The COC extract-treated group showed a significant reduction in infarct size compared to that of I/R group ($p < 0.05$).

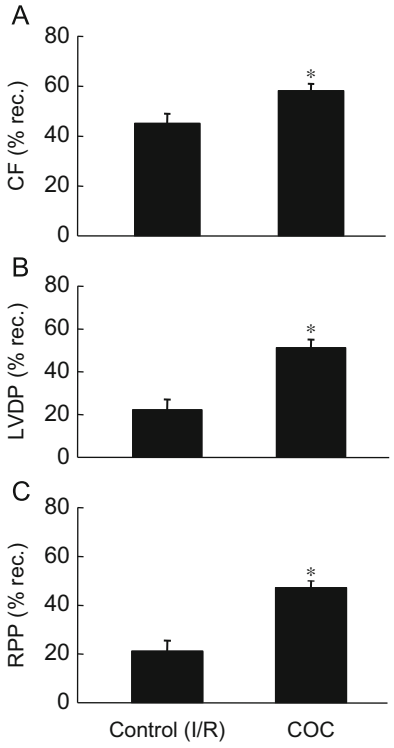

Fig. 3. Effect of COC extract on cardiac contractile and hemodynamics in untreated I/R and COC extract-treated groups. Hearts were subjected to 30 min of global ischemia followed by reperfusion for 45 min. Extract of COC was infused at 1 mg/ml/min for 10 min during the onset of reperfusion. The results show a significant difference (*$p < 0.05$) in (A) CF (B) LVDP and (C) RPP between I/R and COC extract-treated groups.

COC extract regulates molecular and signaling pathways in the reperfused heart

Fig. 6A shows the levels of NAADPH oxidase in the form of gp91, in I/R and COC extract-treated groups. The levels of gp91 were significantly ($p < 0.05$) decreased in the COC extract-treated group when compared to I/R group. Fig. 6B shows the level of xanthine oxidase, which is significantly ($p < 0.05$) reduced following COC extract-treatment compared to I/R group. Fig. 6C shows the level of cytochrome *c* which is significantly ($p < 0.05$) reduced in the COC extract-treated group compared to the untreated I /R group. Fig. 5D shows the level of cleaved caspase-3

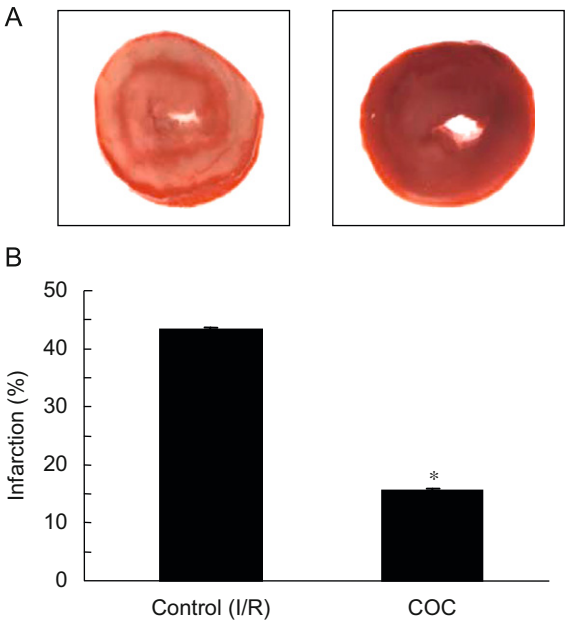

Fig. 5. Effect of COC extract-treatment on I/R-induced myocardial infarction. Irreversible infarction was determined by staining the heart ventricular sections with 1% TTC. The treatment protocol is the same as shown in Fig. 2, except that the reperfusion time was extended to 120 min. (A) Representative photomicrographs of the infarct area of I/R and COC extract-treated hearts, showing infarct (white zones) and non infarct (red zones) after TTC staining. (B) Percentage of infarct area from the left-ventricular area of the sections determined using MetaMorph software. Values are expressed as mean ± S.D. (n=3). The COC extract-treated hearts show a significant reduction in infarction size (*$p < 0.05$) compared to I/R group.

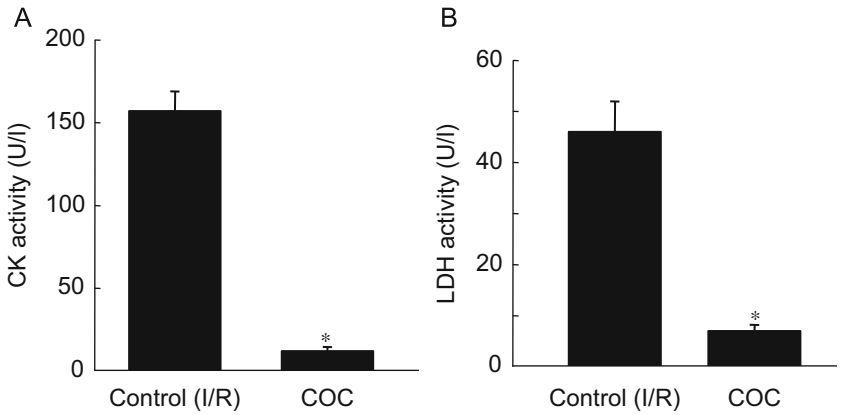

Fig. 4. Effect of COC extract on cardiac enzyme levels. (A) and (B) show the activity of CK and LDH, respectively, in the effluent collected after 15 min of reperfusion. Activity of CK and LDH shows significant reduction (*$p < 0.001$) in the COC extract-treated group, compared to untreated I/R group.

in I/R group and in COC extract-treated hearts. Treatment with COC extract significantly ($p < 0.05$) reduced the levels of cleaved caspase-3 as compared to the untreated I/R group.

Fig. 7A shows the level of Bcl-2 in I/R and COC extract-treated groups. Treatment with COC extract showed a significance increase ($p < 0.05$) in Bcl-2 level compared to I/R group. Fig. 7B shows the level of Hsp70 in I/R and the COC extract-treated hearts. Upon treatment with COC extract, Hsp70 level was increased significantly ($p < 0.05$) as compared to the untreated I/R group.

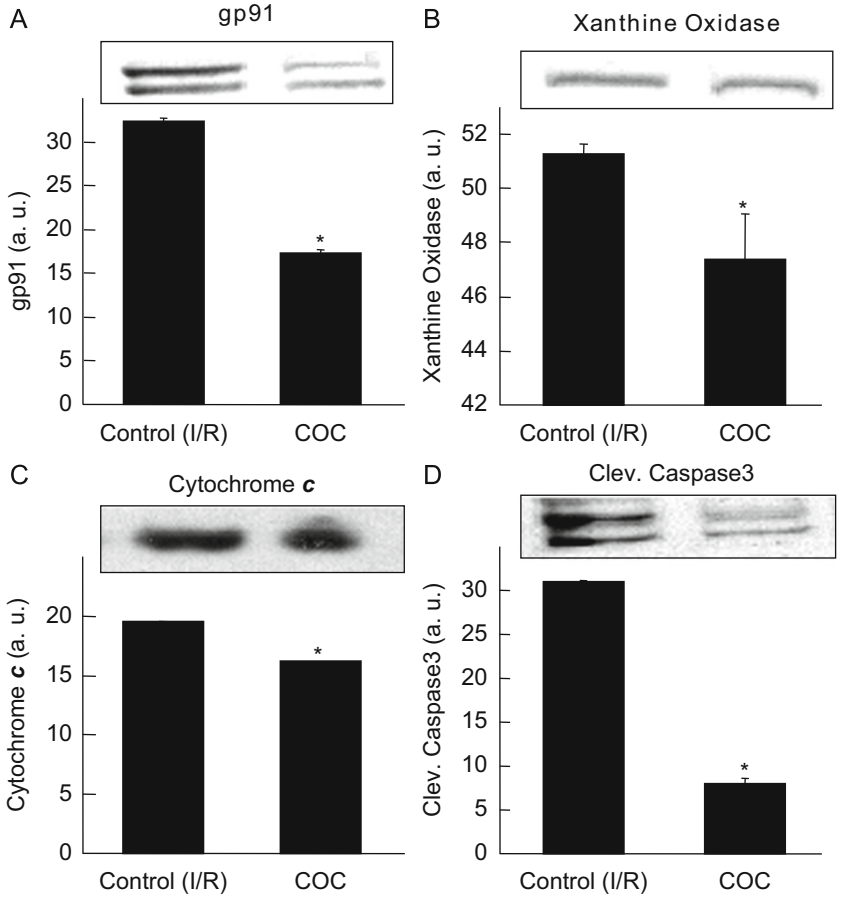

Fig. 6. Effect of COC extract on intracellular organelle-level proteins which are involved in the upregulation of apoptosis. Western blot analysis of (A) gp91 (NADPH oxidase), (B) xanthine oxidase, (C) cytochrome *c*, and (D) cleaved caspase-3, and their respective quantitative analyses (in arbitrary units) for I/R and COC extract-treated hearts. All the proteins were significantly reduced (*$p < 0.05$) in the treated hearts when compared to I/R group.

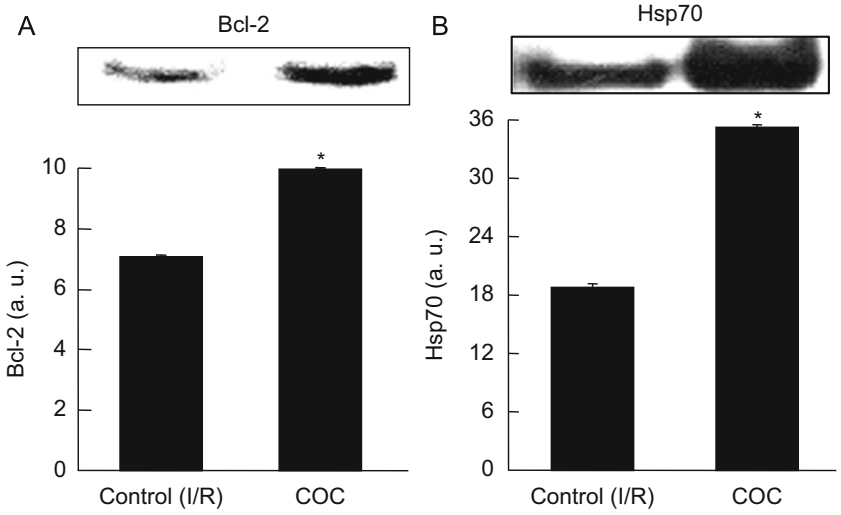

Fig. 7. Effect of COC extract on intracellular organelle level proteins which are involved in the downregulation of apoptosis. Western blot analysis of Bcl-2 (A), Hsp70 (B), and their respective quantitative data in I/R and COC extract-treated heart are shown. Both proteins were significantly increased (*$p < 0.05$) in extract-treated hearts compared to I/R group.

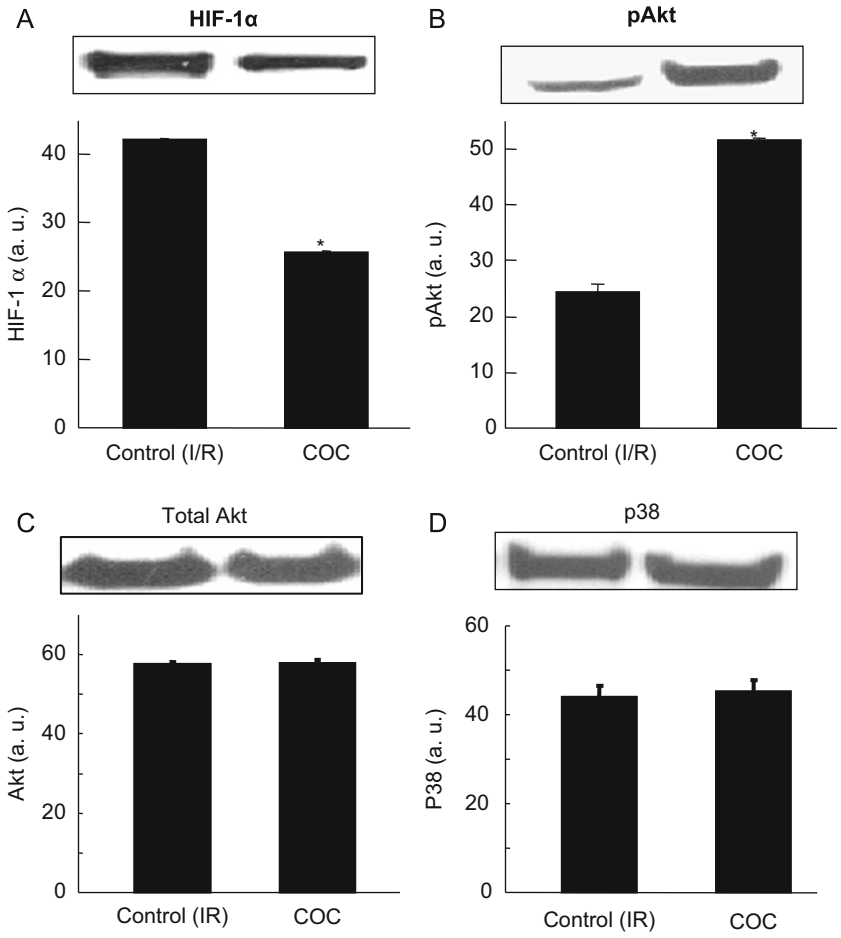

Fig. 8. Effect of COC extract on intracellular signaling proteins. (A) The levels of HIF-1α in I/R and COC extract-treated groups, and their respective quantitative analyses. HIF-1α is significantly reduced (*$p < 0.05$) in the treated group compared to I/R group. (B) shows the levels of phospho-Akt and its quantitative analysis (in arbitrary units). Phospho-Akt is significantly upregulated (*$p < 0.05$) in COC extract-treated group when compared to I/R group. (C) and (D) show the levels of unphosphorylated Akt and phospho-p38 levels, and their quantitative analysis. No significant difference was observed in the expression either of these proteins.

Fig. 8A shows the level of HIF-1α in the untreated I/R and COC extract-treated hearts. Treatment with COC extract significantly ($p < 0.05$) reduced the level of HIF-1α compared to I/R group. Fig. 8B shows the level of phospho-Akt in the control and COC extract-treated groups. The level of phospho-Akt was significantly increased ($p < 0.05$) in the COC extract-treated group compared to that of I/R group. Figs. 8C and 8D show the levels of total Akt and phospho-p38. There was no significant difference between two groups.

Discussion

In the present study, we investigated the effect of a COC extract on I/R-induced cardiac injury. Administration of a COC extract at the onset of reperfusion improved the physiological functions of the heart as a result of the upregulation of anti-apoptotic proteins and downregulation of pro-apoptotic proteins. The I/R-induced injury depresses cardiac function because of the loss of active cardiomyocytes caused by increased free radical production. OPC's present in COC extract might scavenge the free radicals *ex vivo* as demonstrated in the *in vitro* EPR spectroscopy analysis. This reduces the oxidative stress and allows the cells to recover from reperfusion-induced injury. This is achieved by the activation of signaling cascades.

The COC extract was analyzed for its antioxidant activity for the first time using EPR spectroscopy. The $O_2^{\bullet -}$ and $ROO^{\bullet}$ radicals were significantly reduced in the COC extract-treated group demonstrating the antioxidant activity of COC. Previous studies with herbal products in our lab employed the same protocol to study their antioxidant activities by EPR spectroscopy (Khan et al., 2006).

Severe tissue damage is known to occur during reperfusion due to massive production of ROS (Ambrosio et al., 1993). In the present study, administration of COC extract improved the cardiac contractility and left ventricular function, as evidenced by the increase in CF and LVDP. The possible mechanism could be, since the COC extract is available at the time of reperfusion, it can scavenge the free radicals and can reduce the reperfusion-induced injury to the myocardium. The COC extract-treated hearts also showed a four-fold decrease in the infarct size compared to I/R group. Reduction in the damage allows the myocardium to recover faster as reflected in the improved contractile functions of COC extract-treated myocardium. This is further supported by the decreased levels of cardiac marker enzymes CK and LDH indicating less tissue damage in the COC extract-treated myocardium as compared to I/R heart. Our results are in agreement with the pharmacological evaluation of hawthorn extracts which showed a comparable quality profile in 40-70% of aqueous-alcoholic extracting solvents particularly with ethanol or methanol(Vierling et al., 2003).

Recent studies have shown that gp91 is involved mainly in the generation of ROS during the experimental pressure overload, left ventricular hypertrophy, development of contractile dysfunction and interstitial fibrosis (Li et al., 2002; Shen et al., 2006). In our experiment, the levels of gp91 were significantly downregulated in COC extract-treated hearts compared to I/R hearts. A possible explanation could be that administration of COC extract during reperfusion produced a mild negative chronotropic effect, which might have resulted in a reduction of LV pressure leading to a reduction in the pressure overload on cardiomyocytes membrane. This effect may reflect in the reduction in the expression of the gp91 subunit in COC extract-treated I/R group. On the other hand, the ROS produced by the gp91 can be scavenged directly by the OPCs present in the COC extract thereby preventing lipid peroxidation and helping to maintain membrane integrity. This is further supported by the decreased levels of CK and LDH in the treated group. We further observed that the level of XO was significantly reduced in the COC extract-treated hearts compared to I/R group. This could be due to the fact that the OPCs present in the COC extract may normalize the reducing equivalents in the cell by increasing the glutathione (GSH) pool, which can bring back the enzyme into less reactive native form xanthine dehydrogenase.

The levels of cytochrome *c* in the cytosol serve as a marker of mitochondrial damage. During stress conditions like I/R, an increase in ROS and H_2O_2 production, calcium overload, and the change in the mitochondrial membrane potential take place, so that, in turn, there is an increase in the mitochondrial release of cytochrome *c* into the cytosol (Pastorino et al., 1993). Released cytochrome *c* induces apoptosis via the mitochondrial pathway (Green and Kroemer, 1998). In the present study, the level of cytochrome *c* is reduced in the cytosol of the COC extract-treated group compared to I/R group, which could decrease in the oxidative stress in COC extract-treated heart. The possible mechanism could be that the OPCs present in the COC extract scavenged the free radicals and H_2O_2, and protected the mitochondrial membrane from ROS-induced insults.

We also investigated the levels of cardioprotective proteins Bcl-2 and Hsp70. Recent studies showed that Bcl-2 targets the voltage-dependent anionic channel (VDAC) and blocks the release of cytochrome *c*, thereby blocking apoptosis (Shimizu et al., 2000). In the present study, the level of Bcl-2 was significantly upregulated in the COC extract-treated heart compared to I/R group suggesting the protective effect of COC extract. Hsp70, another important protein involved in the protection of the myocardium against reperfusion-induced injury, was also upregulated in the COC extract-treated group. Hsp70 protects the myocardium in different pathways, similar to that of Bcl-2 (Simpkins et al. 1993), by blocking the downstream signaling from cytochrome *c* (Li et al., 2000) and by blocking the NF-κB-mediated apoptosis (Liu et al., 2007).

Although the antioxidant properties of COC extract are well documented in the literature, the upregulation of antiapoptotic proteins by COC has not been studied. In the present study, we observed a significant upregulation of anti-apoptotic proteins in the COC-treated group. Hence, we studied the signaling pathway by which COC extract-treatment can influence antiapoptotic signaling. As this study is based on ischemia/reperfusion-induced injury, we considered the HIF-1α signaling as a leading pathway, followed by a well-documented cell-survival factor like Akt activation and the most common signaling pathway, p38. HIF-1α plays a major role in hypoxia signaling. During hypoxic conditions HIF-1α activates the transcription of hundreds of genes present in the hypoxia responsive element (HRE) (Beck et al., 1991). Transcription of these genes will lead to cell death or cell survival depending upon the severity of hypoxia. The stabilized HIF-1α will lead to the formation of VDAC and release of cytochrome *c* (Denko et al., 2003; Piret et al., 2002; Vengellur et al., 2003). Our results clearly demonstrate that elevation of HIF-1α is well correlated with the release of cytochrome *c* in I/R hearts, which is further supported by the activation of caspase-3. In addition, elevated levels of HIF-1α indirectly show the oxidative stress inside the cell. COC extract-treated hearts showed a significant decrease in HIF-1α levels, thereby providing protection against apoptosis via the cytochrome *c* pathway. This is further supported by the diminished levels of cytochrome *c* and cleaved caspase-3 in COC extract-treated heart.

Akt is involved in many cell survival pathways (Wang et al., 2000). Activated Akt phosphorylates various substrates in the cytosol, and one of its major functions is to act as an antiapoptotic protein indirectly (Parcellier et al., 2008). The level of phosphorylated Akt was increased in the COC-treated group when compared to I/R group. This suggests a positive role of COC extract in preventing apoptosis. Furthermore, it has been clearly demonstrated that elevated phospho-Akt levels inhibit the release of cytochrome *c* from the mitochondria (Parcellier et al., 2008). Similar results were obtained in our experiments. COC extract-treated hearts showed elevated levels of phospho-Akt, but the exact mechanism by which COC extract activates the phosphorylation of Akt is yet to be elucidated. In the present study we also investigated the levels of phospho-p38 which is an important signaling pathway. There were no significant changes observed in the levels of phospho-p38, suggesting that the p38 pathway is not involved following COC extract-treatment.

Conclusion

The overall study shows, COC extract can act as a potent antioxidant, and activator of intracellular signaling which will protect the myocardium during reperfusion. The exact mechanism of the upregulation of the phosphorylation of Akt and downregulation of HIF-1α needs to be elucidated by further studies. In future work, we will further focus on the destabilization of HIF-1α and activation of phospho-Akt by COC extract. In a clinical setting, a patient who has suffered an acute MI may benefit from the administration of this drug at reperfusion in ameliorating the I/R-induced damage.

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