Moutan Cortex Extract Exerts Protective Effects in a Rat Model of Cardiac Ischemia/Reperfusion

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Moutan Cortex Extract Exerts Protective Effects in a Rat Model of
Cardiac Ischemia/Reperfusion

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Abstract

Moutan Cortex (MC) is a traditional Chinese medicine with diverse biological effects. The present study was performed to investigate the effects of MC on myocardial ischemia/reperfusion (I/R) in rats and to explore its possible mechanisms. Sprague-Dawley rats were administered MC extract (1.98 g/kg, i.g.) for 14 days and underwent a subsequent open-chest procedure involving 30 min of myocardial ischemia and 60 min of reperfusion. The cardioprotective effect of MC was demonstrated by reduced infarct size and marked improvement in the histopathological examination. The increase in the activity of superoxide dismutase (SOD) and glutathione (GSH) as well as the reduction of malondialdehyde (MDA) indicated that MC effectively promoted the anti-oxidative defense system. Increased anti-oxidative defense was accompanied by decreased release of lactate dehydrogenase (LDH) and creatine kinase (CK). The reduction in TUNEL-positive myocytes demonstrated that MC decreased myocardial apoptosis. The mRNA expression of B-cell leukemia-2 (Bcl-2) was up-regulated by MC and the ratio of Bcl-2/ Bcl-2-associated X protein (Bax) mRNA expression was increased. MC pretreatment decreased the mRNA expression of inducible nitric oxide synthase (iNOS). The data from this study suggest that MC exerted protective effects on acute myocardial I/R injury via anti-oxidative and anti-apoptotic activities.

Keyword: Moutan Cortex, Ischemia/Reperfusion, cardioprotective, anti-oxidative, anti-apoptotic

1 Introduction

2 The World Health Organization estimates that heart failure initiated by coronary
3 heart disease and myocardial infarction is one of the leading causes of death worldwide
4 (Venugopal et al. 2012). In acute myocardial infarction, coronary reperfusion using
5 percutaneous coronary intervention (PCI) or thrombolysis is the standard therapy for
6 salvaging the myocardium and reducing the onset of heart failure (Gershlick et al. 2013).
7 However, reperfusion itself may lead to accelerated or additional damage, also known
8 as myocardial ischemia/reperfusion (I/R) injury (Dominguez-Rodriguez et al. 2014). No
9 effective therapy is currently available to mitigate the consequences of I/R (Pagliaro and
10 Penna 2014).

11 Moutan Cortex (MC), the root cortex of *Paeonia suffruticosa* Andrews, has been
12 commonly used in traditional Chinese medicine to promote blood circulation and to
13 alleviate blood stasis (Chen et al. 2005). MC extract was reported to exhibit a variety of
14 biological activities, including anti-inflammatory (Fu et al. 2012; Wu and Gu 2009; Yun
15 et al. 2013), anti-allergic (Jiang et al. 2007; Liu et al. 2013), and anti-oxidative effects
16 (Rho et al. 2005; Zhang et al. 2014). Previous studies concerning its anti-oxidative
17 activity indicated that MC extract attenuated oxidative stress in AGE-induced mesangial
18 cell dysfunction and in streptozotocin-induced diabetic nephropathy (Zhang et al. 2014).
19 MC extract also inhibited reactive oxygen species (ROS) production in oxidative-
20 stressed PC12 cells (Rho et al. 2005). Oxidative stress due to ROS is thought to play an
21 important role in I/R injury that impairs cardiac function (Inafuku et al. 2013). ROS are
22 produced in large quantities in the first few minutes of I/R (Dominguez-Rodriguez and

Abreu-Gonzalez 2010). Many herbal antioxidants have been tested for the treatment of I/R injury. Whether the administration of MC, known to possess antioxidant activity, would reduce oxidative stress induced by I/R has not been investigated.

I/R injury are a complex set of events that paradoxically causes tissue injury. There is now growing evidence suggesting a close link between oxidative stress and the induction of apoptosis in the process of I/R (Dobsak et al. 2003). Various studies have demonstrated that both ROS and its oxidation products and other secondary messenger molecules generated by ROS can trigger programmed cell death (Mohanty et al. 2006). In the present experiment, we used an in vivo rat model to study whether MC can attenuate heart injury induced by I/R and the role of oxidative stress and apoptosis in this process.

Materials and methods

Plant material

The plant samples were collected from the tree peony (*Paeonia suffruticosa* Andrews) grown in the Fenghuangshan area in Anhui Province. The samples were kindly provided by Prof. Qinglin Li from Anhui University of Chinese Medicine and were identified by Prof. Keli Chen from the Department of Pharmacognosy, Hubei University of Chinese Medicine. A voucher specimen was preserved in the Key Laboratory of Chinese Medicine Resource and Compound Prescription (Hubei University of Chinese Medicine), Ministry of Education, Wuhan, China.

The MC extract

The dried powdered plant (1.5 kg) was extracted using a tenfold solution of distilled water for 2 h. The extraction was repeated three times. All filtrates were combined and evaporated to dryness under reduced pressure (yield 30.58% w/w). The extract was stored at -20°C until use. The constituents in MC were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and high-performance liquid chromatography (HPLC) (see Supplementary material for details).

Animals

Male Wistar rats (200-250 g) were purchased from Hubei Provincial Center for Disease Control and Prevention, China (certificate No.: SCXK 2008-0005). Animals were maintained in a temperature-controlled room ($22 \pm 2^\circ\text{C}$) and kept on a 12-h light/dark cycle. Food and water were available ad libitum. All rats were allowed to acclimate for a week before testing. Animal care and treatment were approved prior to the study by the Animal Care Committee of Hubei University of Chinese Medicine, Hubei, China.

Surgical procedure

Myocardial I/R was induced as described with minor modifications (Yang et al. 2008). The animals were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg). After insertion of an endotracheal tube, the animals were placed on positive-pressure ventilation at a tidal volume of 10 mL/kg and a rate of 65 strokes/min. A standard limb lead II electrocardiogram (ECG) was monitored with a cardiograph (BL-420S, Chendu Ltd, China) throughout the experiment. A thoracotomy was performed at

the fourth intercostal space, and the pericardium was opened to expose the heart. The left anterior descending (LAD) coronary artery was ligated 3–4 mm from its origin by a 5/0 silk suture with an atraumatic needle. The ends of the ligature were passed through a short length of vinyl tubing. Applying tension to the ligature and pinching off the tube with an arterial clamp resulted in occlusion of the coronary artery and cessation of regional myocardial blood flow. Coronary artery occlusion was maintained for 30 min, after which reperfusion was initiated by withdrawing the vinyl tubing, and the myocardium was reperfused for 60 min. Regional myocardial ischemia was verified by the presence of a zone of cyanosis in the myocardial surface and by a typical elevation of the ST segment in the ECG.

Experimental protocol

Rats were randomly allocated to three groups (n=10): Sham-operated group, I/R group and MC-treated group. Sham-operated and I/R rats were administered 0.9% normal saline for 14 days. In the MC-treated group, the rats were administered MC extract (1.98 g/kg, i.g.) for 14 days. On the 15th day, the rats were subjected to 30-min LAD coronary artery ligation and 60-min reperfusion. Sham-operated rats were subjected to the same surgical procedure, but the artery was not ligated.

Determination of infarct size

At the conclusion of the 60-min reperfusion, the coronary artery was reoccluded, and 2 mL of Evans Blue (2%) was injected slowly through a cannulated right carotid artery. The heart was removed from the chest. The left ventricular area was cut into transverse

sections parallel to the atrioventricular groove from the apex to the base. The slices were then incubated with a 1.5% solution of 2, 3, 5-triphenyltetrazolium chloride (TTC) for 30 min at 37°C to visualize the infarct area. Evans blue-stained areas (the normal left ventricle non-risk region), TTC-stained areas (ischemic but viable tissue), and TTC-negative areas (infarct region) in each slice were cut and weighed. Infarct size is presented as a percentage of the weight of the infarct region to the left ventricle.

Biochemical assays

Blood samples were centrifuged at 3000×g for 10 min, and the sera thus obtained were stored at -80°C until assay. Superoxide dismutase (SOD), malondialdehyde (MDA), glutathione (GSH), lactate dehydrogenase (LDH) and creatine kinase (CK) levels were measured with commercial kits according to the manufacturer's directions (Nanjing Jiancheng Institute of Biotechnology, Nanjing, China).

Histopathological examination

At the end of the experiment, the infarct region of the myocardial tissue was immediately fixed in 10% buffered neutral formalin solution and embedded in paraffin. After processing, wax-embedded sections were cut, stained with hematoxylin and eosin, and examined by light microscopy.

Determination of myocardial apoptosis

To quantify myocardial apoptosis, in situ detection of apoptotic myocytes was performed by using terminal deoxynucleotidyl uridine triphosphate transferase nick-end

labeling (TUNEL) assay as described previously (Ha et al. 2008). Cardiomyocytes from at least five slides per block were randomly selected and evaluated using immunohistochemistry to determine the number and percentage of cells exhibiting positive apoptotic staining under a light microscope. For each slide, five fields were randomly chosen and counted by image-pro plus 6.0. The apoptotic index (AI) is expressed as the number of TUNEL-positive myocytes/the total number of myocytes.

Real-time polymerase chain reaction (PCR)

Total RNA was isolated using Simply P Total RNA Extraction kit (Bioflux, Japan) according to the manufacturer's protocol. Single-strand cDNA was synthesized from 2 µg of RNA by reverse transcription RevertAid™ First strand cDNA Synthesis Kit (Thermo, Maryland, USA). Real-time PCR was performed in an iCycler machine (Bio-Rad, California, USA) using SsoFast™ EvaGreen Supermix (Bio-Rad, Hercules, USA). All PCR primer sequences (Invitrogen Life Technologies, Shang Hai, China) are listed in Table 1. In all cases, melting point analysis revealed amplification of a single product. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard to monitor loading variations. The PCR products were separated on agarose gel and visualized with the Gel Doc-IT™ imaging system (Ultra-Violet Products Ltd., California, USA) in the presence of GelRed™ (Biotium, USA).

Statistical analysis

Values are presented as the means ± S.E.M. Statistical analysis was performed with one-way analysis of variance followed by Bonferroni correction for post hoc t-test (SPSS

Version 17.0 [SPSS, Inc., Chicago, USA]). A P value of less than 0.05 was considered statistically significant.

Results

MC attenuated myocardial infarct size

As shown in Figure 1, the infarct size in myocardial I/R rats was increased significantly compared with the sham group ($P < 0.05$). In comparison with the I/R group, the infarct size in the MC-treated rats was reduced by 32.1% ($P < 0.05$).

MC inhibited I/R-induced oxidative damage

The activities of SOD and GSH were suppressed (20.5% and 54.6%, respectively) in I/R rats relative to the sham group ($P < 0.05$). Furthermore, I/R caused an obvious increase in lipid peroxide by 31.7% ($P < 0.05$). In comparison with the I/R group, the activities of SOD and GSH were significantly increased (16.2% and 73.1%, respectively) in MC-treated rats ($P < 0.01$), and MC significantly reduced MDA levels by 13.4% ($P < 0.05$) (Figure 2).

MC attenuated LDH and CK activities

I/R rats exhibited a remarkable elevation of LDH and CK activities in comparison with the sham group ($P < 0.01$ and $P < 0.05$, respectively). Relative to the I/R group, MC decreased the activity of CK by 30.1% ($P < 0.05$). The LDH level in MC-treated rats was slightly reduced, although no statistical significance was noted (Figure 3).

MC protected histopathological damage

Figure 4 presents representative photomicrographs of each group. The myocardium in the sham group exhibited normal myocardial architecture. However, rat hearts subjected to I/R exhibited myocardial membrane damage, infiltration of inflammatory cells and confluent areas of myonecrosis relative to those in the sham group. MC-treated rats exhibited marked improvement in the degree of myonecrosis, edema and infiltration of inflammatory cells in comparison with the I/R group.

MC attenuated I/R-induced myocardial apoptosis

The TUNEL assay was utilized to analyze the influence of MC on the induction of myocardial apoptosis. As illustrated in Figure 5, few apoptotic cells were observed in the hearts of sham-treated rats. I/R resulted in a significant increase in TUNEL-positive nuclei (AI: $35.4 \pm 3.0\%$, $P < 0.05$ vs. sham group). MC caused a significant reduction in the AI compared with the I/R group (AI: $19.3 \pm 1.6\%$, $P < 0.05$ vs. I/R group).

MC increased the ratio of B-cell leukemia-2/Bcl-2-associated X protein (Bcl-2/Bax) and decreased inducible nitric oxide synthase (iNOS) mRNA expression

Figure 6 presents the PCR result demonstrating the mRNA expression of Bcl-2, Bax and iNOS. A significant decrease was observed in the expression of Bcl-2 mRNA ($P < 0.01$), and increased expression of Bax ($P < 0.05$) and iNOS mRNA ($P < 0.01$) was observed in the I/R group relative to sham-treated rats. Compared with the I/R group, MC increased the mRNA expression of Bcl-2 by 1.05-fold ($P < 0.05$). Although the mRNA expression of Bax was slightly reduced, the ratio of Bcl-2/Bax was increased by

1 1.3-fold. Treatment of MC also caused a remarkable decrease in the mRNA expression
2 of iNOS by 33.9% ($P < 0.05$).

3 **Discussion**

4 The present study was performed to investigate the effects of MC on myocardial I/R
5 and to explore its possible mechanisms. The administration of MC for 14 days
6 significantly reduced the infarct size of I/R rat hearts relative to the sham group. Our
7 studies also demonstrated the cardioprotective effects of MC, as evidenced by marked
8 improvement in the histopathological examination.

9 Oxidative stress has emerged as an important mechanism of myocardial I/R injury.
10 Our findings of depressed SOD and GSH activities and increased MDA level in I/R rats
11 were consistent with earlier reports (Liao et al. 2011; Zhao et al. 2012). SOD is an
12 antioxidant enzyme that catalyzes the conversion of O_2^- into H_2O_2 and O_2 . GSH is
13 another key factor involved in the detoxification of electrophonic metabolites and
14 reactive oxygen intermediates. The decreased activities of SOD and GSH indicate the
15 failure of the antioxidant defense mechanisms to prevent the formation of excessive free
16 radicals (Rodrigo et al. 2013). The excessive free radicals are capable of inducing lipid
17 peroxidation of membranes, altering membrane integrity and increasing membrane
18 fluidity and permeability (Chang et al. 2013). Leakage of a variety of intracellular
19 enzymes, including CK and LDH, from myocardial tissues into blood is an indicator of
20 acute myocardial infarction (Amani et al. 2013). In the present study, the administration
21 of MC restored SOD and GSH activities and inhibited the formation of lipid peroxide
22 products during I/R. The above results indicated that MC effectively restored the anti-

oxidative defense system, thereby protecting the myocardium from I/R injury. Accordingly, I/R-induced elevation of blood CK and LDH was blunted when rats were pretreated with MC.

In the present study, MC reduced myocardial apoptosis, as indicated by a reduction in TUNEL-positive myocytes. Apoptosis plays a crucial role in the development of myocardial infarction and I/R (Ale et al. 2013; Liou et al. 2011; Loan Le et al. 2012), which are related to the pathogenesis of heart failure. Many genes have been reported to be linked to the regulation of programmed cell death under physiological and pathological conditions, and the Bcl-2 and Bax genes are suggested to play a central role (Wang et al. 2013). Since Oltvai et al. provided the first demonstration that the ratio of Bcl-2/Bax determines survival or death following an apoptotic stimulus in 1993 (Oltvai et al. 1993), the role of Bcl-2 and Bax in disease has been extensively studied. Over expression of Bcl-2 in a line of transgenic mice renders the heart more resistant to apoptosis and I/R injury (Chen et al. 2001). Bax-knockout mice exhibit superior tolerance to I/R damage (Ben-Ari et al. 2007; Hochhauser et al. 2003). Bcl-2 and Bax have emerged as potential drug discovery targets for I/R injury (Reed 2006). In the present study, MC pretreatment increased the mRNA expression of the anti-apoptotic gene Bcl-2 while decreasing the mRNA expression of the proapoptotic gene Bax slightly. We suggest that MC might inhibit cardiomyocyte apoptosis by increasing the ratio of Bcl-2/Bax.

We also tested the mRNA expression of iNOS. The iNOS enzyme is responsible for the synthesis of nitric oxide (NO), a free radical that plays a pivotal role in physiology

and pathology in various systems (Chen et al. 2007). iNOS is expressed and activated by stimulating factors during pathologic events (Kanko et al. 2006). NO is a double-edged sword in myocardial I/R and heart failure. Basal levels of NO production from endothelial nitric oxide synthase (eNOS) protect cardiomyocytes from apoptosis, whereas over-production of NO by iNOS promotes apoptosis (Razavi et al. 2005). Excess NO and the co-existence of ROS with NO are injurious to cardiac cells (Otani 2009). In the present study, significantly increased expression of iNOS mRNA was observed in the I/R group, whereas MC caused a remarkable decrease in the mRNA expression of iNOS. We suggest that MC may reduce the excess NO produced during the process of I/R and may exert protective effects. The down-regulation of iNOS reported here is similar to earlier studies demonstrating that MC inhibited iNOS expression in other pathologies, such as lipopolysaccharide- and recombinant interferon-gamma-induced inflammation (Chun et al. 2007; Chung et al. 2007).

In conclusion, the reduction of infarct size and the morphological results demonstrated that MC was effective in the protection of I/R injury. The cardioprotective effect of MC was associated with its anti-oxidative and anti-apoptotic activities. MC might have a therapeutic role in improving clinical outcomes in patients with coronary heart disease.

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Conflict of interest

The authors declare no conflict of interest.

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1 **Table 1.** Primer sequences of B-cell leukemia-2 (Bcl-2), Bcl-2-associated X protein
2 (Bax), inducible nitric oxide synthase (iNOS) and glyceraldehyde-3-phosphate
3 dehydrogenase (GAPDH).

Gene name	Primer sequence (5'-3')
Bax	Forward: TGCAGAGGATGATTGCTGAC
	Reverse: GATCAGCTCGGGCACTTTAG
Bcl-2	Forward: ATACCTGGGCCACAAGTGAG
	Reverse: TGATTTGACCATTTGCCTGA
iNOS	Forward: GGAGAGATTTTTCACGACACCC
	Reverse: CCATGCATAATTTGGACTTGCA
GAPDH	Forward: CGCTAACATCAAATGGGGTG
	Reverse: TTGCTGACAATCTTGAGGGAG

Figure legends

Fig. 1. Pretreatment with Moutan Cortex (MC) extract decreased the infarct size in ischemia/reperfusion (I/R) rats. The data are the means $\pm$ S.E.M. ($n = 10$). $^*P < 0.05$ vs. sham group; $^{\#}P < 0.05$ vs. I/R group.

Fig. 2. The activities of superoxide dismutase (SOD) and glutathione (GSH) were increased by pretreatment with Moutan Cortex (MC) extract in ischemia/reperfusion (I/R) rats, whereas the level of malondialdehyde (MDA) was reduced by MC. The data are the means $\pm$ S.E.M. ($n = 10$). $^*P < 0.05$ vs. Sham group; $^{\#\#}P < 0.01$ vs. I/R group.

Fig. 3. Moutan Cortex (MC) extract attenuated the activity of lactate dehydrogenase (LDH) and creatine kinase (CK) in ischemia/reperfusion (I/R) rats. The data are the means $\pm$ S.E.M. ($n = 10$). $^*P < 0.05$ vs. sham group; $^{**}P < 0.01$ vs. sham group; $^{\#\#}P < 0.01$ vs. I/R group.

Fig. 4. Moutan Cortex (MC) extract protected against histopathological damage in ischemia/reperfusion (I/R) rat hearts stained by hematoxylin and eosin. (A) Sham group, (B) I/R group, (C) MC group.

Fig. 5. Moutan Cortex (MC) extract attenuated myocardial apoptosis in ischemia/reperfusion (I/R) rats. Representative photomicrographs of heart tissues of the sham group (A), I/R group (B) and MC group (C). The apoptotic index (AI) is expressed as a percentage of TUNEL-positive myocytes in tissue sections. The data are the means $\pm$ S.E.M. ($n = 5$). $^{**}P < 0.01$ vs. sham group; $^{\#\#}P < 0.01$ vs. I/R group.

1 **Fig. 6.** Moutan Cortex (MC) extract increased the mRNA expression of B-cell leukemia-
2 2 (Bcl-2) and inducible nitric oxide synthase (iNOS) while decreasing Bcl-2-associated X
3 protein (Bax) mRNA expression slightly in ischemia/reperfusion (I/R) rats.
4 Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal
5 standard. The data are the means $\pm$ S.E.M. (n = 5). * P < 0.05 vs. Sham group; ** P < 0.01
6 vs. Sham group; # P < 0.05 vs. I/R group.

Draft

Fig. 1. Pretreatment with Moutan Cortex (MC) extract decreased the infarct size in ischemia/reperfusion (I/R) rats. The data are the means $\pm$ S.E.M. ($n = 10$). * $P < 0.05$ vs. sham group; # $P < 0.05$ vs. I/R group. 9x6mm (600 x 600 DPI)

Fig. 2. The activities of superoxide dismutase (SOD) and glutathione (GSH) were increased by pretreatment with Moutan Cortex (MC) extract in ischemia/reperfusion (I/R) rats, whereas the level of malondialdehyde (MDA) was reduced by MC. The data are the means $\pm$ S.E.M. (n = 10). *P < 0.05 vs. Sham group; ##P < 0.01 vs. I/R group.
48x12mm (600 x 600 DPI)

Fig. 3. Moutan Cortex (MC) extract attenuated the activity of lactate dehydrogenase (LDH) and creatine kinase (CK) in ischemia/reperfusion (I/R) rats. The data are the means $\pm$ S.E.M. ($n = 10$). * $P < 0.05$ vs. sham group; ** $P < 0.01$ vs. sham group; ## $P < 0.01$ vs. I/R group.
30x11mm (600 x 600 DPI)

Fig. 4. Moutan Cortex (MC) extract protected against histopathological damage in ischemia/reperfusion (I/R) rat hearts stained by hematoxylin and eosin. (A) Sham group, (B) I/R group, (C) MC group. 38x8mm (600 x 600 DPI)

Draft

Fig. 5. Moutan Cortex (MC) extract attenuated myocardial apoptosis in ischemia/reperfusion (I/R) rats. Representative photomicrographs of heart tissues of the sham group (A), I/R group (B) and MC group (C). The apoptotic index (AI) is expressed as a percentage of TUNEL-positive myocytes in tissue sections. The data are the means $\pm$ S.E.M. (n = 10). **P < 0.01 vs. sham group; ##P < 0.01 vs. I/R group. 85x43mm (600 x 600 DPI)

Fig. 6. Moutan Cortex (MC) extract increased the mRNA expression of B-cell leukemia-2 (Bcl-2) and inducible nitric oxide synthase (iNOS) while decreasing Bcl-2-associated X protein (Bax) mRNA expression slightly in ischemia/reperfusion (I/R) rats. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard. The data are the means $\pm$ S.E.M. (n = 5). *P < 0.05 vs. Sham group; **P < 0.01 vs. Sham group; #P < 0.05 vs. I/R group.
63x23mm (600 x 600 DPI)