Treatment with Cardiotonic Pills® after Ischemia-Reperfusion Ameliorates Myocardial Fibrosis in Rats

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ABSTRACT

Objective: The present study was designed to evaluate whether CP was beneficial in alleviating myocardial fibrosis following I/R injury.

Methods: Sprague–Dawley rats were subjected to 30 minutes occlusion of the LADCA, followed by reperfusion. CP (0.4 or 0.8 g/kg) was daily administered starting from three hour after reperfusion until day 6. Coronary venular diameter, RBC velocity, albumin leakage, MBF, heart function, myocardial infarction and fibrosis size, myocardium ultrastructure, MPO activity, and MDA level were measured. The expression of MCP-1, RP S19, TGF-β1, P-Smad3, Smad4, MMP-9 and α-SMA, and the infiltration of leukocytes were examined.

Results: CP post-treatment ameliorated I/R-induced myocardial RBC velocity reduction, MBF decrease, cardiac dysfunction, and albumin leakage increase. Moreover, myocardial infarction and fibrosis size, MPO activity, MDA level, the expression of RP S19, TGF-β1, P-Smad3, Smad4, MMP-9 and α-SMA, the number of CD68-positive cells increased significantly after I/R, and myocardium collagen deposition was observed on day 6 after reperfusion. All the alterations after I/R were significantly ameliorated by CP.

Conclusions: Post-treatment with CP ameliorates I/R-induced myocardial fibrosis, suggesting that CP may be applied as an option for preventing cardiac remodeling after I/R injury.

Key words: chemokines, cardiac microcirculation, myocardial infarction, leukocytes

Abbreviations used: –dp/dtmax, left ventricular maximum descent velocity; +dp/dtmax, left ventricular maximum upstroke velocity; CP, cardiotonic pills; DLA, 3,4-dihydroxy-phenyl lactic acid; FITC, fluorescein isothiocyanate; HR, heart rate; I/R, ischemia/reperfusion; LADCA, left anterior descending coronary artery; LVDP, left ventricular diastolic pressure; LVEDP, left ventricular end diastolic pressure; LVPSP, left ventricular systolic pressure; MBF, myocardial blood flow; MCP-1, monocyte chemotactic protein-1; MDA, malondialdehyde; MMP-9, matrix metalloproteinase-9; MPO, myeloperoxidase; PN, Panax notoginseng; RBC, red blood cell; RP S19, ribosome protein S19; SAB, Salvianolic acid B; SM, Salvia miltiorrhiza; TGF-β1, transforming growth factor-β1; TTC, 2,3,5-triphenyltetrazolium chloride; α-SMA, alpha-smooth muscle actin.

INTRODUCTION

Interventional therapies have currently been applied widely to salvage patients’ lives to some extent, but they do not reduce the risk of death [1]. This is because the abrupt reperfusion of ischemic myocardium causes tissue damage that may eventually lead to myocardial fibrosis [9]; the latter may further increase the risk of incidence of heart failure and cardiac arrhythmias [32]. Therefore, therapies that target toward ameliorating myocardial fibrosis induced by I/R are expectedly beneficial in improving the outcome of patients.

Myocardial fibrosis is the sequel of a cascade of reactions, preceded with myocardial microcirculatory disturbances and inflammatory response, followed by myocardial injury [22]. Subsequently, damaged cardiac muscle releases several chemokines to affect monocyte recruitment and infiltration [4], which is a vital process in the formation of myocardial fibrosis. Besides the classical chemokines, such as MCP-1 [14], RP S19 dimer is another type of monocyte...

chemotactic factor, the monocyte chemotactic capacity of which has been clearly demonstrated in rheumatoid arthritis synovial lesion [20] and atherosclerotic vascular lesion [27]. On the other hand, chemokine-mediated recruited monocytes may create a fibrogenic environment through secretion of a wide variety of pro-fibrotic factors into the injured myocardial tissue [3]. However, whether RP S19 dimer is critical in I/R-induced recruitment of monocytes is unknown. TGF-β1 has long been believed to be the most important pro-fibrotic cytokine that stimulates cardiac fibroblasts via activating Smad2/3 signaling pathway to produce collagen protein and modulate the fibrosis process [26]. To prevent myocardial fibrosis, the regimes that have been tested in animal models mostly target for TGF-β1 [15]. This limited option necessitates new treatments as alternatives to improve outcome of the patients with the risk of undergoing myocardial fibrosis.

CP is a compound Chinese medicine consisting of SM, PN, and Borneol. It was approved in 1994 by the China Food and Drug Administration for treating ischemic angina pectoris, and passed Phase II clinical trials by the US Food and Drug Administration in 2010. Our previous studies have demonstrated that pre-treatment with CP could attenuate cardiac microcirculation dysfunction by I/R [36]. Proteomic study on protein targets against I/R injury revealed that combination of SM and PN showed a better cardioprotective effect than SM or PN alone [35], suggesting superiority of CP. Some of the effective compounds of CP have been isolated, and the biochemical structures of which have been identified, such as DLA and SAB from SM [7,18], and panax notoginseng saponins from PN [29]. Evidence from clinical medicine revealed that the major ingredients of CP are effective for pectoris [12,30]. In addition, increasing research showed that both of SM and PN exhibit antioxidative ability [6,34], and antifibrotic properties. For example, SAB has been reported to perform antifibrotic activity through inhibition MAPK and Smad signaling in TGF-β1-stimulated hepatic stellate cells [19]. PN and its effective elements were reported as capable of attenuating hepatic fibrosis [5]. However, it is unclear whether post-treatment with CP may protect damaged myocardium from developing myocardial fibrosis following I/R.

In the present study, we intended to investigate the effects of CP post-treatment on I/R-induced rat myocardial fibrosis, and to explore the underlying mechanism.

**MATERIALS AND METHODS**

**Animal Model**

Male Sprague–Dawley rats weighing 240–260 g were randomly assigned to Sham, I/R 3h, I/R 6d, I/R 6d + CP 0.4, and I/R 6d + CP 0.8 groups. The number of animals in each group and for various parameters is detailed in Table 1. Anesthesia of rats was brought about initially by intraperitoneal injection of sodium pentobarbital (60 mg/kg) and maintained by 1% isoflurane inhalation. Intraoperative monitoring of adequate anesthesia was achieved by a pinch on the toe. A positive pressure respirator (ALC-V8; Shanghai, China) was used for artificial respiration. The chest was opened between the second and fourth ribs, and the LADCA was ligated with a 5/0 silk. After 30 minutes of coronary artery occlusion, ligation was released for reperfusion [16,36]. Laser Doppler blood flow measurement was used to verify I/R [25]. In the Sham group, animals underwent all

<p>| Table 1. Number of animals for different experimental groups and various parameters |
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<table>
<thead>
<tr>
<th>Groups</th>
<th>Venular diameter, RBC velocity, albumin leakage</th>
<th>MBF, TTC staining</th>
<th>Mallory staining, IHC</th>
<th>Western blotting</th>
<th>MPO activity</th>
<th>Ultrastructure assay</th>
<th>Heart function, ELISA, α-SMA</th>
<th>Total</th>
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<tr>
<td>Sham</td>
<td>6</td>
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<td>4</td>
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<td>32</td>
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<td>I/R 3h</td>
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<td>I/R 6d + CP 0.4</td>
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<td>I/R 6d + CP 0.8</td>
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<td>160</td>
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The same animals were used for determination of venular diameter, RBC velocity, and albumin leakage, and so were for determination of MBF and TTC staining. For both Mallory staining and IHC examination, the paraffin sections were derived from the same animals. For Western blotting, tissue specimens were collected from four animals in each group. Three animals in each group were used for MPO activity and ultrastructure examination. Heart function assay, ELISA for MDA, MCP-1, RP S19, and IHC for α-SMA used the same animals. Sham: Sham group; I/R 3h; I/R 3h group; I/R 6d: I/R 6d group; I/R 6d + CP 0.4: I/R 6d plus post-treatment with CP 0.4 g/kg group; I/R 6d + CP 0.8: I/R 6d plus post-treatment with CP 0.8 g/kg group.
surgical procedures, except that the silk passing around LADCA was not tied. Chest was closed and the animal was allowed to recover. For post-operative analgesia, buprenorphine (0.1 mg/kg, sc) was administered every 12 hours for 48 hours. All animals were purchased from the Animal Center of Peking University Health Science Center (Beijing, certificate NO. SCXK 2002-0001), and handled according to the guidelines of the Peking University Health Science Center Animal Research Committee. The investigations conformed to the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, 1996), and were approved by Peking University Biomedical Ethics Committee Experimental Animal Ethics Branch (LA2010-001).

Drug Administration
CP (batch number: 200605027) was obtained from Tasy Pharmaceutical Co, Ltd (Tianjin, China), one pill containing 9 mg of SM, 1.76 mg of PN, 0.5 mg of Borneol, and 13.74 mg of polyethylene glycol [25]. For the animals in the post-treatment with CP groups, CP dissolved in 2-mL saline was given every 24 hours through a gavage tube starting from three hours after reperfusion until day 6 at 0.4 g/kg (I/R 6d + CP 0.4 group) or 0.8 g/kg (I/R 6d + CP 0.8 group), the doses 4.9-fold and 9.8-fold higher, respectively, than that administered to human in clinic. Animals in the Sham and I/R groups received equivalent volume of saline in the same way. One and a half hour after the last administration of CP or saline on day 6, animals were anesthetized with ketamine/xylazine (130 mg ketaminel/mL and 20 mg xylazine/mL) by intraperitoneal injection, and underwent surgical procedure. Heart was removed for determination of the variables below. The survival rates of animals on day 6 in the I/R 6d group, I/R 6d + CP 0.4 group, and I/R 6d + CP 0.8 group were 86.2%, 92.6%, and 96.2%, respectively.

Diameter of and Red Blood Cell Velocity in Coronary Venules
Microvessels with diameter of 25–40 μm in the region dominated by the third branch of LADCA were selected and observed under an upright microscope (BX51WI; Olympus, Tokyo, Japan) using an epi-illuminating light. The microvessel images were recorded for four seconds using a DVD videocassette recorder (DVR-560H; Philips, Amsterdam, Netherlands) through a high-speed video camera system (Fastcam-ultra 1000i; Photron, Tokyo, Japan) at a rate of 500 frames per second, and the images were transmitted to a monitor (20PF5120; Philips). Then, the recordings were replayed at a rate of 25 frames per second from the stored images, so that the microcirculation dynamic state in a cardiac cycle could be observed clearly. The diameter of venules and RBC velocity in the phase of end-systole were detected using Bio-image analysis system (Image-Pro Plus 6.0 software; Media Cybernetics, MD, USA). Using this software, RBC in the axial flow in a venule was selected and the time it spent to travel a distance of 100 μm was determined; the RBC velocity was thus calculated [8].

FITC-Albumin Leakage from Coronary Venules
FITC-conjugated albumin (Sigma-Aldrich, St. Louis, MO, USA) was infused (50 mg/kg) through femoral vein after the observation of microcirculation dynamics. Five minutes after FITC-albumin injection, venular images were acquired using a SIT camera (EB-CCD Camera C7190; Hamamatsu, Shizuoka, Japan) via an upright fluorescence microscope (DM-LFS; Leica, Solms, Germany) under irradiation at wavelength of 455 nm. The fluorescence intensities of FITC-albumin inside the lumen of the selected coronary venules (Iv) and in the surrounding interstitial area (Ii) were estimated using Image-Pro Plus 6.0 software. The ratio of Ii/Iv was calculated as an indication for albumin leakage [7].

Myocardial Blood Flow
Images of MBF supplied by LADCA were determined using Laser-Doppler Perfusion Imager (PeriScan PIM3; Perimed, Stockholm, Sweden) equipped with a computer by evaluation of the areas of 3 × 4 mm² with the software LDPlwin 3.1 (Perimed), and results were expressed as percentage of the Sham [36].

Heart Function Test
A cannulation was inserted into the left ventricle through the right carotid artery, which was connected to a bio-function experiment system BL-420F (Chengdu Taimen Technology Ltd, Chengdu, China). HR, LVSP, LVDP, LVEDP, +dp/dtmax, and −dp/dtmax were measured six days after reperfusion with BL-420F equipment.

Myocardial Infarct Size
Hearts were rapidly excised and sliced parallel to the atrioventricular groove into five sections (1-mm thick each) from the apex cordis to the ligation site. Slices were incubated for 15 minutes at 37°C in a 0.375% solution of TTC (AMRESCO, OH, USA), and then photographed as a digital image at ×10 magnification by a digital camera (Digital Sight DS-5M-U1; Nikon, Tokyo, Japan). Myocardial infarct size was analyzed using Image-Pro Plus 6.0 software and expressed as a percentage area of the whole slice [36].

Myocardial Tissue MPO Activity
Rat hearts were excised at a 4-mm level above the apex cordis. Heart tissue was homogenized, and MPO activity was determined using an MPO assay kit (Jiancheng Bioengineering Institute, Nanjing, China). One unit of MPO activity is defined as the quantity of enzyme degrading 1 μmol peroxide/min at 37°C. The change in absorbance was measured spectrophotometrically at 460 nm. The data were expressed as MPO activity per gram of wet tissue.
MDA Level in Myocardial Tissue

The level of MDA in the myocardium was measured as an indicator of lipid peroxidation. Heart tissue was dissected from the surrounding infarction areas of the left ventricle six days after reperfusion and homogenized. The MDA level was determined according to a Rat MDA ELISA Kit’s instruction (DZE30626; RD, Huanya Biomedicine Technology Co, Ltd, Beijing, China).

Myocardial Fibrosis and Immunohistochemistry

Rat hearts were excised as above and fixed in 4% paraformaldehyde. Serial paraffin sections (5-μm thickness) were prepared. Mallory staining was used for studying myocardial fibrosis [25]. We calculated the ratio of blue area to the area of whole section at ×10 magnification with Image-Pro Plus 6.0 software as the ratio of myocardial fibrosis size. Sections for immunohistochemistry were incubated overnight at 4°C with specific antibody against MCP-1 (Santa Cruz, CA, USA), RP S19, CD68 (ED1; Abcam, Cambridge, MA, USA), MPO (Thermo Scientific, CA, USA), α-SMA (Abcam), or MMP-9 (Santa Cruz). Then specific antibody was detected by incubation with an HRP-conjugated secondary antibody and revealed using the DAB Substrate Kit. PBS was used, instead of primary antibody, as negative control. Photomicroscopy was performed at ×200 magnification. Five visual fields were selected from each section for analysis of the protein expression using Image-Pro Plus 6.0 software, and the mean density of RP S19 or the mean number of CD68-positive cells was determined.

The Level of MCP-1 and RP S19 in Plasma

Plasma was collected using heparin as an anticoagulant at three hours, 24 hours, and on day 6 after reperfusion, and then samples were centrifuged for 15 minutes at 3000 rpm at 4°C. The supernatant was removed, and the level of MCP-1 and RP S19 was detected using a Rat MCP-1 ELISA Kit (DZE30495; Huanya Biomedicine Technology Co, Ltd) and a Rat RP S19 ELISA Kit (E02R0076; Blue Gene Biototechnology Co, Ltd, Shanghai, China) by microplate reader (MULTISKAN MK3; Thermo).

Western Blotting Assay

Myocardial tissues were taken from the infarction surrounding areas of the left ventricle. Total protein was extracted using a protein extraction kit (Applygen Technologies) and mixed with 5 × electrophoresis sample buffer. After electrophoresis, the separated proteins were transferred to polyvinylidene difluoride membrane. After being blocked with 5% nonfat dry milk, the membrane with target proteins was incubated overnight at 4°C with an antibody against TGF-β1, P-Smad3, Smad4, RP S19, or MMP-9. The blots were incubated with a respective HRP-conjugated second antibody, and then immunoreactive bands were revealed using an enhanced chemiluminescence system. The protein signal was quantified by scanning densitometry in the X-film by Image-Pro Plus 6.0 software. The result of each group was expressed as relative optical density compared with that from the Sham group [36].

Ultrastructure Examination

An approximately 1 mm² fresh myocardial tissue block was cut at a 4-mm level above the apex cordis from the region of left ventricle. Tissues were fixed with 3% glutaraldehyde and post-fixed with 1% osmium tetroxide. The specimens were processed as routine for ultrathin sections. Sections were stained with uranyl acetate and lead citrate, and ultrastructural changes were then evaluated by using a transmission electron microscope (JEM-1230; Tokyo, Japan) [36].

Statistical Analysis

All parameters were expressed as means ± SE. Statistical analysis was performed using one-way ANOVA, followed by Bonferroni test for multiple comparisons. A probability less than 0.05 was considered statistically significant [33].

RESULTS

Changes in Myocardial Infarct Size

Representative heart slices stained by TTC are illustrated in Figure 1A. The white area in myocardial tissue represents the infarction region. Apparently, myocardial tissue slices...
from the Sham group exhibited no infarct. In contrast, noticeable infarct areas were observed in myocardial tissue slices in both I/R 3h and 6d groups, and such myocardial infarct was reduced obviously in myocardial tissues from the groups receiving post-treatment with CP. Quantitative analysis of the infarct size further confirms the above results, suggesting that CP exerts therapeutic effects on I/R-induced myocardium infarct in a dose-dependent manner (Figure 1B).

Heart Function
Heart function was assessed, and the result is shown in Figure 2. In comparison with the Sham group, I/R caused a significant decline in LVSP and +dp/dtmax, and an increment in LVDP, LVEDP, and −dp/dtmax, indicating an impairment on heart function. Obviously, this impairment was alleviated by post-treatment with CP.

Histology and Myocardial Fibrosis
Figure 3 displays the myocardium histomorphological images by Mallory staining for evaluation of myocardial fibrosis in various groups. Compared with the Sham group (a1, b1), distinct alterations occurred in the myocardial tissues from the I/R groups. In the I/R 3h group, the damage to myocardium presented as disruption of myocardial fibers and neutrophil infiltration (a2, b2), whereas ischemia for 30 minutes followed by reperfusion for six days gave rise to prominent myocardial fibrosis where plenty of extracellular matrix collagen could be detected (a3, b3). I/R-induced myocardium fibrosis was reduced significantly by post-treatment with CP in a dose-dependent manner (a4-a5 and b4-b5), as confirmed by a quantitative analysis (B).

Figure 4 presents the representative electron micrographs of the myocardium in the five groups. The myocardium in the Sham group retained the characteristics of normal structure with regularly arranged and densely packed myofibrils and mitochondria (A). The I/R challenge for three hours provoked a dramatic injury in cardiac muscle cells (B), as indicated by disrupted myofibrils and mitochondria hyperplasia. On the other hand, exposure to I/R for six days elicited a remarkable alteration in myocardium, manifested as extensive existence of collagen fibers, in addition to myofibril degeneration and fragmentation (C). I/R challenge also provoked emigration of leukocytes from vessel (B, C). These changes were alleviated by post-treatment with CP, especially with 0.8 g/kg CP (E).

![Figure 2](image)

**Figure 2.** Effect of CP on rat cardiac function. (A, B, C, D, E, F) The changes in HR, LVSP, +dp/dtmax, LVDP, LVEDP, and −dp/dtmax in various groups. **Sham:** Sham group; **I/R 3h:** I/R 3h group; **I/R 6d:** I/R 6d group; **I/R 6d + CP 0.4:** I/R 6d plus post-treatment with CP 0.4 g/kg group; **I/R 6d + CP 0.8:** I/R 6d plus post-treatment with CP 0.8 g/kg group. Data are mean ± SE. *p < 0.05 vs. Sham group, **p < 0.05 vs. I/R 6d group.
Expression of TGF-β1 and Related Signaling Molecules

TGF-β1 has been known for its role in myocardial fibrosis. Therefore, we investigated the expression of TGF-β1 and related signaling molecules in myocardial tissue by western blotting. As shown in Figure 5A, the expression of TGF-β1 was significantly increased after three hours of reperfusion, as compared with the Sham group, and such TGF-β1 upregulation was maintained at the same level until six days of reperfusion. Post-treatment with CP at the dose of 0.8 g/kg ameliorated the I/R-induced increase in TGF-β1 expression apparently. The expression of P-Smad3 and Smad4 exhibited a comparable pattern to that of TGF-β1 (Figure 5B, C).

Diameter of, RBC velocity in, and Albumin Leakage from, Coronary Venules, and Myocardial Blood Flow

With the high-speed video camera, microvessels in a beating heart were clearly visible for assessment of microcirculatory dynamics. There was no significant difference in venular diameter among the five groups (Figure 6A, B). As shown in Figure 6C, there were marked decreases in RBC velocity in both I/R 3h and I/R 6d groups, compared with the Sham group. By contrast, post-treatment with CP at a dose of either 0.4 or 0.8 g/kg significantly attenuated the I/R-induced decrease in RBC velocity.

Post-treatment with CP also ameliorated FITC-labeled albumin leakage from coronary venules evoked by I/R, and...
representative images are illustrated in Figure 6A. No albumin leakage was noticed in the Sham group (A1), whereas apparent leakages were observed in the rats from the I/R 3h and I/R 6d groups (A2, A3), which were abated by post-treatment with CP at the dose of 0.4 g/kg (A4) and 0.8 g/kg (A5). Albumin leakage from the coronary venules

Figure 5. Effect of CP on the expression of TGF-β1 and related signaling molecules. (A, B, C) The Western blotting results of TGF-β1, P-Smad3, and Smad4, respectively, with the corresponding densitometry that was normalized to expression in Sham group. Sham: Sham group; I/R 3h: I/R 3h group; I/R 6d: I/R 6d group; I/R 6d + CP 0.4: I/R 6d plus post-treatment with CP 0.4 g/kg group; I/R 6d + CP 0.8: I/R 6d plus post-treatment with CP 0.8 g/kg group. Data are mean ± SE. *p < 0.05 vs. Sham group, #p < 0.05 vs. I/R 6d group.

Figure 6. Effect of CP on venular diameter, RBC velocity, albumin leakage, and MBF. (A) Representative images of albumin leakage. The rectangular boxes on A2 represent the areas selected for determination of fluorescence intensities in venular lumen and interstitial area. A1: Sham group; A2: I/R 3h group; A3: I/R 6d group; A4: I/R 6d plus post-treatment with CP 0.4 g/kg group; A5: I/R 6d plus post-treatment with CP 0.8 g/kg group. Bar = 100 μm. (B, C) Quantitative evaluation of venular diameter and RBC velocity. (D) Quantitative evaluation of the results of albumin leakage presented as percent change. (E) Representative color images of heart acquired by Laser–Doppler Perfusion Imager. E1: Sham group; E2: I/R 3h group; E3: I/R 6d group; E4: I/R 6d plus post-treatment with CP 0.4 g/kg group; E5: I/R 6d plus post-treatment with CP 0.8 g/kg group. (F) Quantitative evaluation of MBF. Sham: Sham group; I/R 3h: I/R 3h group; I/R 6d: I/R 6d group; I/R 6d + CP 0.4: I/R 6d plus post-treatment with CP 0.4 g/kg group; I/R 6d + CP 0.8: I/R 6d plus post-treatment with CP 0.8 g/kg group. Data are mean ± SE. *p < 0.05 vs. Sham group, #p < 0.05 vs. I/R 6d group.
was quantified, as shown in Figure 6D. In the I/R 3h and I/R 6d groups, albumin leakage from coronary venules increased significantly compared with the Sham group. Post-treatment with CP significantly attenuated the I/R-induced increase in albumin leakage from coronary venules.

Figure 6E shows MBF color images acquired by Laser–Doppler Perfusion Imager in each group. The MBF was decreased in both I/R 3h and I/R 6d groups (E2, E3), compared with the Sham group (E1). Post-treatment with CP obviously attenuated I/R-induced decrease in MBF. The above results were confirmed by quantitative evaluation, as shown in Figure 6F. MBF in the I/R 3h and I/R 6d groups was significantly decreased in comparison with the Sham group. Post-treatment with CP significantly ameliorated the I/R-induced MBF decreases in a dose-dependent manner.

MPO Activity and MDA Level in Myocardium

As a marker enzyme of neutrophils, MPO expression in myocardial tissue was assessed by immunohistochemistry and MPO activity assay, and the results in different conditions are shown in Figure 7A, B. Only a few cells exhibited MPO-positive staining in the Sham group (A1). Thirty minutes of ischemia followed by three hours of reperfusion evoked a significant increase in infiltration of neutrophils (A2). On day 6 of reperfusion, the number of MPO-positive cells declined when compared with that at three hours of reperfusion, but it remained larger than that in the Sham group (A3). Nonetheless, the I/R-induced increase in the number of MPO-positive cells in myocardium was diminished significantly by post-treatment with CP 0.8 g/kg (A5). This immunohistochemistry result for MPO was confirmed by MPO activity assay (Figure 7B).

To evaluate the antioxidant capacity of CP, MDA level in myocardial tissue was assessed. As shown in Figure 7C, the content of MDA increased significantly after I/R, as compared with the Sham group. Post-treatment with CP apparently restrained the increase in MDA level evoked by I/R.

MCP-1 Level in Myocardium and Plasma

As a classical chemotactic factor, the MCP-1 level was evaluated by immunohistochemistry and ELISA. As shown in Figure 8, MCP-1 expression in myocardium and the plasma MCP-1 content increased significantly after I/R, while on both of which, CP had no apparent effect.

Release of RP S19

To gain insight into the mechanism underlying the amelioration action of CP on I/R-evoked myocardial injury and RP S19 release, immunohistochemistry, western blotting study and ELISA were carried out for RP S19, and the result is presented in Figure 9. RP S19 dimer is released mainly by apoptotic cells, and it attracts monocyte migration [21]. There was no observable RP S19 dimer staining in the Sham group (A1). However, the expression of RP S19 dimer was apparent in the I/R 3h group (A2), and further augmented six days after reperfusion (A3). By contrast, the abnormal upregulation of RP S19 dimer was significantly diminished by post-treatment with CP (A4 and A5). As shown in A6, RP S19 was mainly located in myocardial cells. Quantitative evaluation for optical density of RP S19 in different groups confirmed the survey result (B). The plasma RP S19 level in different groups is shown in Figure 9C; when compared with the Sham group, I/R challenge dramatically increased RP S19 content. Of note, post-treatment with CP could restrain the increase in RP S19 from 24 hours to six days after reperfusion.

Infiltration of Monocytes

Infiltration of monocytes is a critical step in the development of I/R-induced cardiac injury. Immunohistochemistry staining for CD68 is shown in Figure 10A, B to display monocyte infiltration. Only a few cells exhibited CD68-positive in the Sham group and in the I/R 3h group (A1 and A2). In
contrast, the number of CD68-positive cells increased prominently six days after reperfusion (A3), in line with an increase in the expression of RP S19. Post-treatment with CP at both 0.4 and 0.8 g/kg blunted monocyte infiltration significantly (A4, A5 and B).

Expression of MMP-9 and a-SMA
Immunohistochemistry and western blotting were conducted for assessment of MMP-9 expression, and the results are shown in Figure 11A, C. The expression of MMP-9 was rarely detected in the Sham group, but increased apparently after reperfusion, especially at three hours. Quantitative evaluation for optical density of MMP-9 showed that the I/R-induced increase in MMP-9 expression was significantly diminished by post-treatment with CP.

Transformation of myocardial fibroblast into myofibroblast promotes formation of myocardial fibrosis, and a-SMA serves as a marker of myofibroblast. As shown in
Figure 11B, only vascular smooth muscle exhibited \(\alpha\)-SMA-positive in the Sham and I/R 3h groups. On the Contrary, there were lots of \(\alpha\)-SMA-positive cells in myocardial tissue six days after reperfusion, while post-treatment with CP at both 0.4 and 0.8 g/kg inhibited \(\alpha\)-SMA expression prominently.

DISCUSSION

The major finding of the present study is that post-treatment with CP could inhibit post-infarction myocardial fibrosis induced by I/R. At three hours after reperfusion, we found obvious occurrence of microcirculation disturbance, cardiac dysfunction, and myocardium damage, manifested as a pronounced decrease in RBC velocity and myocardium blood flow, and an increase in albumin leakage, neutrophil infiltration and infarct size. On day 6, the left ventricle revealed an attenuated microcirculation disturbance in terms of RBC velocity, myocardium blood flow, and albumin leakage, but without significance, and a decrease in neutrophil infiltration. On the other hand, myocardium by then displayed considerable infiltration of macrophages, transition of fibroblasts to myofibroblasts, and plenty of collagens, indicating myocardial fibrosis. Impressively, administration of CP significantly abated the post-infarction myocardial fibrosis induced by I/R, along with the reduction in RP S19 dimer production, macrophages infiltration, and decrease in MMP-9 expression and the number of myofibroblasts.
Infiltration of monocytes is a pivotal episode in the initiation of myocardial remodeling after I/R [24], because the monocytes recruited in the infarct region may differentiate into macrophages and participate in the healing process through production of growth factors, such as TGF-β1 [2]. TGF-β1 is considered to promote the activation of fibroblasts to elaborate collagen, and regulation of extracellular matrix by synthesis of matrix metalloproteinases like MMP-9 [28]. The transition of fibroblasts to myofibroblasts plays an important role in the cardiac remodeling process, which is regulated by MMP-9, and SA-B is reported to be a competitive blocker of MMP-9, and could inhibit myofibroblast transformation from fibroblast [31]. Consistent with others [25], the present study showed a marked infiltration of monocytes and upregulation of α-SMA in myocardium after I/R. Furthermore, we found a remarkable increase in RP S19 dimer production in I/R-challenged myocardium, and that CP was able to blunt RP S19 dimer release, monocytes migration, TGF-β1/Smads signaling molecules expression, MMP-9 expression, fibroblasts activation, and subsequent collagen deposition.

RP S19 dimer is produced and released mainly by apoptotic cells, and known to recruit monocytes from the circulation to the apoptotic lesion [11]. Considering our previous study that CP was demonstrated to reduce myocardial infarct size and apoptotic cells, and improve myocardial structure [36], it is reasonable to propose that the protective effect of CP on the myocardium from I/R injury accounts for the decrease of RP S19 dimer, which may, in turn, diminish monocyte migration and infiltration and thus inhibit cardiac remodeling. The results of the present study not only provide a framework for understanding the mechanism by which CP attenuates I/R-elicted post-infarction myocardial fibrosis but also suggest RP S19 dimer as a potential target for developing novel prophylaxis regime for the patients who are at risk of undergoing myocardium fibrosis after I/R.

The beneficial effect of CP on myocardial microcirculation disturbance may contribute to the inhibitory action of CP on myocardial fibrosis. In the present study, infiltration of neutrophils was revealed at three hours after reperfusion, which would last six days after I/R in the absence of CP. We showed in a previous publication that the accumulation of neutrophils plays a vital role in the initiation and development of microcirculatory disorders by plugging blood flow and impacting endothelium, and CP pretreatment protected from neutrophil recruitment by inhibition of adhesion molecules on both neutrophils and endothelial cells [36]. It is most likely that the same mechanism underlies the action of CP on neutrophil infiltration observed in the present study, and it is the attenuation of microcirculation disturbance by CP that reduces RP S19 dimer production and macrophage infiltration, thus preventing myocardium from development of fibrosis. Furthermore, emigrated neutrophils may exacerbate myocardial injury through releasing ROS and MPO [10,13], resulting in myocardium damage, RP S19 dimer production, and monocyte migration, which ultimately lead to myocardial fibrosis.

The antioxidative ability of CP may be one of the mechanisms behind its therapeutic benefits on myocardial microcirculation dysfunction and myocardial fibrosis after I/R. In line with this presumption, several recent publications from ours and others show that CP and its major components are indeed capable of attenuating microcirculatory disturbance and fibrosis, and that this potential is correlated mainly, if not solely, with its antioxidative ability [6,7]. For example, SA-B has been reported to be effective against fibrosis and I/R injury, possibly through its antilipid peroxidation action [17]. PN suppresses gut I/R-induced fibrogenesis through reduced generation of lipid peroxides [23]. Likewise, the present study revealed that CP could inhibit MDA production evoked by I/R. However, further works are necessary to explore the role of CP antioxidant potential in attenuation of I/R-elicted myocardial fibrosis.

In conclusion, the results of the present study support the clinic use of CP as a management for the patients after interventional therapies, and provide novel information for better understanding the mechanism behind the beneficial role of CP.

PERSPECTIVE

I/R injury caused by interventional therapies may induce myocardial fibrosis, and even evoke arrhythmia and heart failure, leading to sudden death. Presumably, therapies that target toward ameliorating myocardial fibrosis induced by I/R are expectedly beneficial in improving the outcome of patients. In the present study, we found that CP, a traditional Chinese medicine for treating ischemic angina pectoris, could prevent myocardial fibrosis induced by I/R. This finding provides an experimental basis for clinical use of CP to improve the outcome of patients after interventional therapies.

ACKNOWLEDGMENTS

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REFERENCES


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