Cardiotonic pills, a compound Chinese medicine, protects ischemia-reperfusion-induced microcirculatory disturbance and myocardial damage in rats

Zhao N, Liu YY, Wang F, Hu BH, Sun K, Chang X, Pan CS, Fan JY, Wei XH, Li X, Wang CS, Guo ZX, Han JY. Cardiotonic pills, a compound Chinese medicine, protects ischemia-reperfusion-induced microcirculatory disturbance and myocardial damage in rats. Am J Physiol Heart Circ Physiol 298: H1166–H1176, 2010. First published January 29, 2010; doi:10.1152/ajpheart.01186.2009.—Cardiotonic pills (CP) is a compound Chinese medicine widely used in China, as well as other countries, for the treatment of cardiovascular disease. However, limited data are available regarding the mechanism of action of CP on myocardial function during ischemia-reperfusion (I/R) injury. In this study, we examined the effect of CP on I/R-induced coronary microcirculatory disturbance and myocardial damage. Male Sprague-Dawley rats were subjected to left coronary anterior descending branch occlusion for 30 min followed by reperfusion with or without pretreatment with CP (0.1, 0.4, or 0.8 g/kg). Coronary blood flow, vascular diameter, velocity of red blood cells, and albumin leakage were evaluated in vivo after reperfusion. Neutrophil expression of CD18, malondialdehyde, inhibitor-kBα, myocardial infarction, endothelial expression of intercellular adhesion molecule 1, apoptosis-related proteins, and histological and ultrastructural evidence of myocardial damage were assessed after reperfusion. Pretreatment with CP (0.8 g/kg) significantly attenuated the I/R-induced myocardial microcirculatory disturbance, including decreased coronary blood flow and red blood cell velocity in arterioles, increased expression of CD18 on neutrophils and intercellular adhesion molecule 1 on endothelial cells, and albumin leakage from venules. In addition, the drug significantly ameliorated the I/R-induced myocardial damage and apoptosis indicated by increased malondialdehyde, infarct size, myocardial ultrastructural changes, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling-positive myocardial cells, inhibitor-kBα degradation, and expression of Bcl-2, Bax, and caspase-3 in myocardial tissues. The results provide evidence for the potential role of CP in preventing microcirculatory disturbance and myocardial damage following I/R injury.

I/R exerts multiple insults in the microcirculation that are often manifested as endothelial cell dysfunction, enhanced adhesion of leukocyte, macromolecular efflux, production of reactive oxygen species, and mast cell degranulation, which are followed by decreased coronary blood flow (24). The release of ROS and myocardium damage are exaggerated by leukocyte activation and emigration from microvessels. Moreover, the production of ROS has been demonstrated to be a crucial event in the degradation of inhibitor-kBα (IkBa), and the activation and translocation to nucleus of nuclear factor-kB (NF-kB), resulting in the expression of various adhesion molecules and apoptosis factor (14, 35). It has been reported that I/R-elicited myocardial apoptosis is mediated, at least in part, by caspase-3, Bcl-2, and Bax (6). Although ischemic preconditioning is known to protect the myocardial tissue from I/R injury (3), the clinical application of such an approach is limited for obvious ethical and practical reasons. On the other hand, pharmacological agents that produce the pathophysiological responses mimicking preconditioning (prophylactic) have been considered as alternative means for reducing ischemic insults. Several cardioprotective drugs, including ROS scavengers, calcium channel blockers, adenosine, and nicorandil, have recently been evaluated in clinical trials; their protective effects are insufficient, however (21). One of the reasons for these unsatisfactory results may reside in the multifactorial mechanisms of reperfusion injury and the lack of therapy that can simultaneously target multiple pathways (21).

Cardiotonic pills (CP) is a pharmaceutical preparation consisting of ingredients extracted from Salvia miltiorrhiza (SM), Panax notoginseng (PN), and Borneol. It has been widely used in China, Korea, Russia, Cuba, and Vietnam for the prevention and management of ischemic heart diseases. Increasing studies have been published evaluating the pharmacology of SM and PN. Recent research demonstrates that SM increases myocardial capillary density and reduces infarct size and peroxide production after myocardial infarction in rats (23, 26). Our laboratory has previously demonstrated that pretreatment with 3,4-dihydroxy-phenyl lactic acid, a major ingredient of SM aqueous extract, improves microcirculatory function by reducing peroxide production, leukocyte adhesion, and albumin leakage in rat mesentery following I/R (9). Another component of CP, PN, is known to protect the brain and liver from ischemic injury, which is ascribed to its inhibitory effect on inflammation by attenuating tumor necrosis factor (TNF-α) production and Bcl-2, caspase-1, and caspase-3 expression (25). Pretreatment with ginsenoside Rb1, a major ingredient of PN, is able to attenuate myocardial I/R injury, an effect partly...
mediated by activating the phosphatidylinositol 3-kinase/AKT-dependent pathway. Furthermore, evidence is accumulating that supports the beneficial effect of CP in treating microcirculatory disturbance and cell damage imposed by I/R or other insults. Our previous study demonstrated that CP could inhibit gut I/R-induced adhesion of leukocytes to hepatic sinusoid, the activation of platelets and leukocytes, and the formation of thrombus (11, 31). Other studies show that CP can inhibit photochemical reaction-induced venous thrombosis in the rat mesentery by reducing the expression of adhesion molecules, such as CD31, in platelets and vascular endothelial cells. CP is also reported to ameliorate TNF-α-induced apoptosis in the myocardium (31). Besides, there are numerous clinical trials on CP for the treatment of angina pectoris (37). However, no study has been published regarding the use of CP as a prophylactic drug to prevent cardiac microcirculatory disturbance and myocardial damage induced by I/R.

The purpose of this study was to investigate whether pretreatment with CP improves coronary microcirculation and reduces myocardial damage on I/R challenge. The potential mechanisms underlying its protective effects were examined.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats, weighing 240–260 g, were purchased from the Animal Center of Peking University Health Science Center (Beijing, certificate no. SCXK 2002–0001). The animals were fasted for 12 h before the experiment, while allowing free access to water. All animals were handled according to the guidelines of the Peking University Health Science Center Animal Research Committee, and the surgical procedures and experimental protocol were approved by Peking University Biomedical Ethics Committee Experimental Animal Ethics Branch.

Drugs. CP (batch number: 200605027) was obtained from Tasly Pharmaceutical (Tianjing, China), which was prepared from water-ethanol extract of SM, PN, and borneol under the guidelines of Good Manufacturing Practice and Good Laboratory Practice verified by the Chinese, Australian, and US Government agencies. The concentration of the major compounds for quality control of the CP was analyzed by HPLC (18). One pill of CP contains 9 mg of SM, 1.76 mg of PN, 0.5 mg of borneol, and 13.74 mg of polyethylene glycol.

Animal model and drug administration. Rats were anesthetized with urethane (1.25 g/kg) by intramuscular injection. After tracheotomy, the animals were ventilated with a positive-pressure respirator (ALC-V8, Shanghai, China). The chest was opened between the second and fourth ribs by left thoracotomy. The heart was quickly exposed, and a 3–0 silk ligature was placed around the left coronary artery (1–2 mm region under the boundary of pulmonary artery pyramid and left auricle of heart). A small polyethylene tube was placed between the ligature and myocardial tissues. For the I/R group, the coronary artery was occluded by tightening the ligature for 30 min, and reperfusion was achieved by releasing the tension applied to the ligature (24a). In the sham group, the animals underwent the same surgical procedures, except that the silk passing around the left coronary artery was not tied. In the CP + I/R group, CP dissolved in saline was given through intragastric administration 90 min before ischemia at the volume of 4 ml/kg, with the final concentration of 0.1, 0.4, and 0.8 g/kg, which were 1.2-, 4.9-, and 9.9-fold higher than that administered to humans in the clinic, respectively, for the three CP pretreatment groups. A total of 195 animals were included and randomly distributed into the sham, I/R, CP (0.1 g/kg) + I/R, CP (0.4 g/kg) + I/R, and CP (0.8 g/kg) + I/R groups, with 39 animals in each group. See Table 1 for further details.

Diameter of coronary arterioles and venules and RBC velocity in the vessels. The hemodynamics of coronary microvessels (25–40 µm in diameter) was observed using an upright microscope (BX51WI, Olympus) connected with a high-speed video camera (APX, Photon Fastcam-ultimate) under epi-illumination. The heart was kept warm and moist by continuous superfusion with saline solution at 37°C, but without infusion with any solution. Images were transmitted onto a monitor (20PF5120, Philips) and DVD videocassette recorder (DVR-560H, Philips). The red blood cell (RBC) velocity in the venule and arteriole was recorded for 4 s at a rate of 500 frames/s, and the stored images were replayed at a rate of 25 frames/s. The RBC velocity and vessel diameter were determined using Image-Pro Plus 5.0 software (Media Cybernetic) before ischemia (baseline), 30 min after ischemia, and 30 and 60 min after reperfusion, respectively. Results were expressed as percentages of the baseline (10).

FITC-albumin leakage from coronary venules. FITC-albumin (Sigma, 50 mg/kg) was administrated via the femoral vein at 60 min after reperfusion. Fluorescent images were acquired under an excitation light (455-nm wavelength) that was irradiated from a mercury burner (100 W) to an upright fluorescence microscope (DM-LFS, Leica, Germany) at 2–3 min after injected FITC-albumin. The fluorescent intensity in the venule and extravascular interstice was measured with Image-Pro Plus 5.0 software (Media Cybernetic). The ratio of fluorescent intensity outside and inside the venule was calculated (16).

Myocardial blood flow. After left thoracotomy, myocardial blood flow (MBF) was measured by using Laser-Doppler Perfusion Imager (PeriScan PIM3, Perimed, Sweden) equipped with a computer before and after ischemia and 5, 10, 30, 60 min after reperfusion. All images were evaluated with the software LDPIwin 3.1 (Perimed, Sweden). Results were expressed as percentages of the baseline (36).

Expression of CD18 on neutrophils. Sixty minutes after reperfusion, 10 ml blood were collected via the abdominal aorta and anticoagulated with 3.8% sodium citrate. To determine the expression of CD18 on neutrophils, 50 µl blood were taken and incubated with 1 µg...
FITC-labeled antibody against CD18 (BD) for 20 min at room temperature in the dark. RBCs were lysed with hemolysin (BD), and the samples were washed twice with PBS. The mean fluorescence intensity of CD18 was accessed with a flow cytometer (FACS Calibur, BD). Neutrophils were then sorted by characteristic forward/side-scatter expression, as reported. Five thousand neutrophils were evaluated for each sample (27).

Malondialdehyde level in myocardial tissue. The level of malondialdehyde (MDA) in the myocardium was measured as an indicator of lipid peroxidation. Animals were killed at 60 min of reperfusion. The myocardial tissue was dissected from the surrounding infarction areas of the left ventricle and homogenized in ice-cold buffer (0.1 mM C12H22O11, 1.5 M MgCl2, 50 mM Tris) with a weight-to-volume ratio of 1:9. The homogenate was centrifuged at 8,000 rpm for 15 min at 4°C. The MDA level was determined according to the manufacturer’s instruction (Nanjing Jiancheng Institute of Biotechnology). Protein concentration was measured by the Coomassie blue protein-binding assay using bovine serum albumin as a standard (32).

Myocardial infarct size. Sixty minutes after reperfusion, the heart was rapidly excised and sliced parallel to the atrioventricular groove into five sections (1 mm thick) from the apex of the ligation site. The myocardial slices were incubated for 15 min at 37°C in a 0.375% solution of 2,3,5-triphenyltetrazolium chloride in phosphate-buffered saline, and then photographed as digital images (Digital Sight DS-U1, Nikon). Total infarct area was analyzed by Image-Pro Plus 5.0 software (Media Cybernetic). Myocardial infarct size was expressed as a percentage of the left ventricle (33).

Myocardial damage. Ninety minutes after reperfusion, the heart was cut from approximately the middle one-third, between the apex and the ligation point, and fixed through perfusion with 4% paraformaldehyde. The fixed tissues were embedded in OCT and cut into 6-μm sections. Damaged cardiac myocytes were detected by using a cell

---

**Fig. 1.** The effect of cardiotonic pills (CP) pretreatment on red blood cell’s (RBC) velocity in coronary arteriole (CA) and venule (CV) in rat after ischemia-reperfusion (I/R). A: the cardiac coronary microcirculation. Bar = 100 μm. B: the time course of change in RBC’s velocity in CA. C: the time course of change in RBC’s velocity in CV. Sham, sham group; I/R, I/R group; CP 0.1 + I/R, pretreatment with CP at 0.1 g/kg plus I/R group; CP 0.4 + I/R, pretreatment with CP at 0.4 g/kg plus I/R group; CP 0.8 + I/R, pretreatment with CP at 0.8 g/kg plus I/R group. Values are means ± SE from 6 animals. *P < 0.05 vs. sham group. #P < 0.05 vs. I/R group.

**Fig. 2.** The effect of pretreatment with CP on FITC-albumin leakage from rat CV wall 60 min after reperfusion. A: the fluorescence images of albumin leakage from the rat CV wall. Bar = 50 μm. A1: sham group; A2: I/R group; A3: CP 0.1 g/kg + I/R group; A4: CP 0.4 g/kg + I/R group; A5: CP 0.8 g/kg + I/R group. B: the quantification of albumin leakage from rat CV wall in various groups. Values are means ± SE from 6 animals. *P < 0.05 vs. sham group. #P < 0.05 vs. I/R group.
death detection kit (Roche). The total number of nuclei (blue) and the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) positive nuclei (green) in each field were observed using a laser confocal microscope system (Radiance2100, Bio-Rad; Axiovert 200, Zeiss). Five fields were selected from the surrounding infarction areas of the left ventricle, and the number of TUNEL-positive nuclei was automatically calculated with Image-Pro Plus 5.0 software (Media Cybernetic) (28).

Histological and immunohistochemistry evaluation of myocardial tissues. Hearts were removed from approximately the middle one-third between the apex and the ligation point at 90 min after reperfusion, fixed in 4% formalin, and further prepared for paraffin sectioning. The paraffin sections (5 μm) were either stained with hematoxylin and eosin, or incubated with an antibody against ICAM-1 (BD), caspase-3 (Santa Cruz), Bcl-2 (Santa Cruz), or Bax (NOVUS) overnight before being blocked with bovine serum albumin. The samples were then incubated with a biotinylated secondary antibody followed by avidin-biotin-peroxidase complex. Positive staining was visualized with diaminobenzidine. The images were captured by a digital camera connected to a microscope (BX512DP70, Olympus). Five fields were selected from the surrounding infarction areas of the left ventricle, and the mean optical density was measured using Image-Pro Plus 5.0 software (Media Cybermetrics) (2).

Myocardial ultrastructure. Fresh myocardial tissues (2 mm³) were excised from the surrounding infarction areas of the left ventricle at 90 min after reperfusion. Tissues were fixed with 3% gluteraldehyde and postfixed with 1% osmium tetroxide. The specimens were processed for ultrathin sections. The sections were stained with uranyl acetate and lead citrate, and the ultrastructural changes of myocardial tissue were then evaluated by a transmission electron microscope (BX512DP70, Olympus). Five fields were selected from the surrounding infarction areas of the left ventricle, and the mean optical density was measured using Image-Pro Plus 5.0 software (Media Cybermetrics) (2).

Western blot assay for myocardial tissue IkBα. At 60 min after reperfusion, 100-mg myocardial tissue sample was taken from the surrounding infarction areas of the left ventricle and stored at −80°C before use. Whole protein was extracted by protein extraction kit (Applygen Technologies) and mixed with 2× electrophoresis sample buffer. After electrophoresis on 10% polyacrylamide gels, the separated proteins were transferred from the gels to polyvinylidene difluoride membrane. The membrane with target protein was scissored and incubated with the antibody against IkBα (Calbiochem) after being blocked with 5% nonfat dry milk. The membrane was then washed and incubated with a horseradish peroxidase-conjugated second antibody. The membranes were analyzed using the enhanced chemiluminescence system, according to the manufacturer’s protocol and exposure in the dark box. The protein signal was quantified by scanning densitometry in the X-film by bio-image analysis system (Image-Pro plus 5.0, Media Cybermetrics). The results from each experimental group were expressed as relative integrated intensity compared with that from the sham group (12).

Statistics. All parameters are expressed as means ± SE. Statistical analysis was performed using one-way ANOVA, followed by Tukey test for multiple comparisons. A probability of <0.05 was considered to be statistically significant.

RESULTS

Diameter of, and RBC velocity in, coronary arterioles and venules. In the beating heart, coronary microvessels and RBC moving inside the vessels were clearly observed under the microscope equipped with a high-speed video camera (Fig. 1A). At baseline, the diameters of the coronary arterioles of the sham group, I/R group, CP 0.1 g/kg + I/R group, CP 0.4 g/kg + I/R group, and CP 0.8 g/kg + I/R group were 15.25 ± 1.26, 15.56 ± 0.41, 15.63 ± 0.92, 16.17 ± 1.01, and 15.75 ± 1.52 μm, and the coronary venules were 44.67 ± 1.13, 46.2 ± 1.98, 46.03 ± 2.78, 46.25 ± 2.28, and 45.42 ± 0.93 μm, respectively. There was no significant difference in diameters at baseline of either coronary arterioles or venules among the five groups. No significant alteration in the diameter of either coronary arterioles or venules was observed in all groups over the period of examination (data not shown).

The time course of RBC velocity change in coronary arterioles is illustrated in Fig. 1B. In the sham group, there was no significant change in RBC velocity in arterioles throughout the observation period. In the I/R group, RBC velocity in arterioles significantly decreased in the beginning of reperfusion and gradually recovered thereafter. Pretreatment with CP at the dose of 0.8 g/kg significantly attenuated the I/R-induced RBC
velocity decrease from 30 min after reperfusion, whereas no effect was observed for lower doses of CP on this alteration.

Figure 1C shows the time course of change in RBC velocity in coronary venules. Similar to that in coronary arterioles, the RBC velocity in coronary venules in the sham group remained nearly constant throughout the observation period. By contrast, the RBC velocity in venules was significantly decreased in the I/R group immediately after reperfusion and remained at a low level until 60 min of reperfusion. Pretreatment with CP at 0.8 g/kg, but not at 0.4 and 0.1 g/kg, significantly attenuated the I/R-elicited RBC velocity decrease from 30 min after reperfusion.

Albumin leakage from coronary venules. Pretreatment with CP prevented FITC-labeled albumin leakage from coronary venules evoked by I/R, with representative images illustrated in Fig. 2A. No albumin leakage was detected in the sham group (A1), whereas apparent leakage was observed in the I/R group (A2), which was abated by pretreatment with CP (0.8 g/kg) (A5).

The leak response in different groups was quantified, as shown in Fig. 2B. Compared with the sham group, the I/R group showed a significantly increased albumin leakage (A3), which was significantly decreased by pretreatment with CP (0.8 g/kg) (A5).

Fig. 5. The influence of pretreatment with CP on the expression of ICAM-1 on vascular endothelial cells in myocardium of rats after I/R. A: representative photomicrographs of immunohistochemistry staining for ICAM-1. A1: the location of the sample for ICAM-1 immunostaining; A2: sham group; A3: I/R group; A4: CP 0.1 g/kg + I/R group; A5: CP 0.4 g/kg + I/R group; A6: CP 0.8 g/kg + I/R group. Bar = 100 μm. Arrow indicates ICAM-1-positive vascular endothelium. B: quantitative analysis of ICAM-1-positive staining in various groups. Values are means ± SE from 6 animals. *P < 0.05 vs. sham group. #P < 0.05 vs. I/R group.

Fig. 6. Influence of pretreatment with CP on malondialdehyde (MDA) level in the myocardial tissue of rats subjected to I/R. Values are means ± SE from 6 animals. *P < 0.05 vs. sham group. #P < 0.05 vs. I/R group.

Fig. 7. The effect of pretreatment with CP on myocardial infarct size of rats after I/R. A: representative slice of left ventricle (LV) stained by 2,3,5-triphenyltetrazolium chloride in sham group (a), I/R group (b), CP 0.1 g/kg + I/R group (c), CP 0.4 g/kg + I/R group (d), and CP 0.8 g/kg + I/R group (e). The infarct myocardium is revealed as pale white. B: the quantitative analysis of infarct size in various groups presented as percentage of LV. Values are means ± SE from 6 animals. *P < 0.05 vs. sham group. #P < 0.05 vs. I/R group.
group displayed significant increase in extravasation of albumin from coronary venules at 60 min after reperfusion. Pre-treatment with CP attenuated the I/R-induced albumin leakage from coronary venules in a dose-dependant manner, although a significant effect was observed only for the 0.8 g/kg CP group.

Changes in MBF. Figure 3A shows the color images acquired by the Laser-Doppler Perfusion Imager in five groups at different time point, where the different magnitude of MBF is indicated by distinct color, with the red color representing the highest MBF. No obvious difference in MBF at baseline was observed among the five groups (a1, b1, c1, d1, and e1). As expected, however, manipulation of ischemia gave rise to an apparent decrease in MBF (b2, c2, d2, and e2) compared with the sham group (a2). This decrease in MBF was recovered to a great extent during reperfusion in the 0.8 g/kg CP pretreatment group (e3 and e4), whereas no significant recovery was observed by pretreatment with CP at 0.1 g/kg (c3 and c4) or 0.4 g/kg (d3 and d4).

The time courses of MBF changes in the five groups are plotted in Fig. 3B. As noticed, in the I/R group, the MBF decreased to 65% of baseline after ischemia, which was reversed to 82% of baseline from 5 to 10 min after reperfusion and then gradually reduced to 77% of baseline at the end of 60 min of observation. MBFs in CP pretreatment groups followed a time course similar to that in the I/R group, but with significant difference in that MBF was reversed from 106 to 99% of baseline from 5 to 30 min after reperfusion in 0.8 g/kg CP pretreatment and to 91% of baseline 5 min after reperfusion in 0.4 g/kg CP pretreatment group, presenting a beneficial action on the MBF disorders evoked by I/R.

Expression of CD18 on neutrophils. The expression of the adhesion molecule CD18 on neutrophils was determined by flow cytometer 60 min after reperfusion; the result is presented as fluorescence intensity in Fig. 4. The fluorescence intensity of CD18 was enhanced by I/R, and the increased CD18 expression was significantly attenuated by pretreatment with 0.8 g/kg of CP, but not a lower dose of CP (0.4 or 0.1 g/kg).

ICAM-1 expression on the endothelium of coronary blood vessels. Figure 5A shows the representative images of immunohistochemistry staining of ICAM-1 in myocardial tissues in various conditions. Positive staining of ICAM-1 was obvious on the endothelium surface of myocardial blood vessels in the I/R group (A3), as well as in 0.1 and 0.4 g/kg of CP pretreatment groups (A4 and A5). In contrast, 0.8 g/kg of CP pretreatment apparently attenuated the I/R-induced expression of ICAM-1 (A6).

A quantitative evaluation of ICAM-1 expression on the endothelium of myocardial blood vessels in different groups is presented in Fig. 5B. After I/R, ICAM-1 expression in myocardial tissues was significantly higher than that in the sham group.

Fig. 8. The effects of pretreatment with CP on myocardial injury. A: terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-stained myocardium of rats from sham (A1), I/R (A2), pretreatment with CP at 0.1 g/kg + I/R (A3), pretreatment with CP at 0.4 g/kg + I/R (A4), and pretreatment with CP at 0.8 g/kg + I/R (A5) group. Arrows indicate TUNEL-positive cells. Arrowheads indicate normal nuclei. Bar = 50 μm. B: representative photomicrographs of immunohistochemistry staining for caspase-3 of rat myocardium in sham (B2), I/R (B3), pretreatment with CP at 0.1 g/kg + I/R (B4), pretreatment with CP at 0.4 g/kg + I/R (B5), and pretreatment with at CP 0.8 g/kg + I/R (B6) group. Arrows indicate caspase-3-positive staining. Bar = 100 μm. B1: the location of the samples for caspase-3 immunohistochemistry staining. C: the quantitative analysis of TUNEL-positive cells. D: quantitative analysis of caspase-3 activity. Values are means ± SE from 6 animals. *P < 0.05 vs. sham group. #P < 0.05 vs. I/R group.
group, and the increased expression was significantly reduced by pretreatment with CP at the dose of 0.8 g/kg. Lower dose (0.1 or 0.4 g/kg) of CP did not show any effect on ICAM-1 expression on the endothelium.

**MDA level in the myocardium.** To address the tissue oxidative damage, the MDA level in surrounding infarction areas of the left ventricle myocardial tissues was accessed 60 min after reperfusion. As shown in Fig. 6, the level of MDA observed at 60 min of reperfusion was significantly increased in the I/R group compared with the sham group. Pretreatment with all three doses of CP reduced the I/R-enhanced MDA production with statistic significance.

**Myocardial infarct size.** Slices of the hearts from the various groups were stained by 2,3,5-triphenyltetrazolium chloride for evaluation of myocardial infarct size, and the representative images are illustrated in Fig. 7A. As expected, myocardial tissue slice from the sham group exhibited no evidence of infarction (Fig. 7A, a). In contrast, noticeable infarct areas were observed in myocardial tissue slice in the I/R group (Fig. 7A, b), which was reduced in the animals receiving CP pretreatment, particularly at 0.8 g/kg (Fig. 7A, e). A quantitative analysis of the infarct area further confirms the result (Fig. 7B).

**Tissue damage and apoptosis-related protein expression in myocardial tissues.** To determine the effect of pretreatment with CP on myocardial apoptosis after I/R, experiments were conducted on myocardium 90 min after reperfusion by virtue of TUNEL staining and immunohistochemistry of caspase-3, Bcl-2, and Bax. Figure 8A shows the images of TUNEL staining in surrounding infarction areas of the left ventricle myocardium from the various groups. In the sham group, few TUNEL-positive cells were seen in the myocardium (A1). In contrast, a large number of TUNEL-positive cells were observed in the I/R group (A2). Noticeably, the number of TUNEL-positive cells in the myocardium of CP + I/R groups was decreased at all of the three doses tested (CP 0.1, 0.4, and 0.8 g/kg; A3, A4, and A5, respectively). Figure 8C is the statistical results of TUNEL-positive cells in surrounding infarction areas of the left ventricle myocardium from various groups, which is consistent with the qualitative survey.

The immunohistochemistry and corresponding quantification for caspase-3 in surrounding infarction areas of the left ventricle myocardial tissues from the five groups are shown in Fig. 8, B and D, respectively. A large number of caspase-3-positive cells were observed in myocardial tissues in the I/R group (B3), which is in distinct contrast to that in the sham group, and the expression was significantly reduced by pretreatment with CP at the dose of 0.8 g/kg. Lower dose (0.1 or 0.4 g/kg) of CP did not show any effect on caspase-3 expression.

**Fig. 9.** The effect of pretreatment with CP on the expression of Bcl-2 and Bax in myocardium of rats after I/R. A: representative photomicrographs of immunohistochemistry staining for Bcl-2 (A) and Bax (B) in sham (2), I/R (3), pretreatment with CP at 0.1 g/kg + I/R (4), pretreatment with CP at 0.4 g/kg + I/R (5), and pretreatment with CP at 0.8 g/kg + I/R (6) group. A1: the location of sample for Bcl-2 immunohistochemistry staining. Bar = 100 μm. B1: the location of sample for Bax immunohistochemistry staining. Arrows indicate immunohistochemistry staining positive cells. Arrowheads indicate nuclei of cardiac muscle cells. C: quantitative analysis of Bcl-2 expression in rat myocardium in different groups. D: quantitative analysis of Bax expression in rat myocardium in different groups. Values are means ± SE from 6 animals. *P < 0.05 vs. sham group. #P < 0.05 vs. I/R group.
group (B2). The number of caspase-3-positive cells was decreased by pretreatment with CP at 0.8 g/kg (B6). CP at 0.1 or 0.4 g/kg did not show any effect (Fig. 8D).

Figure 9 illustrates the expression of apoptosis-related proteins Bcl-2 and Bax in surrounding infarction areas of the left ventricle myocardial tissues. As noticed from Fig. 9, A and C, the expression of Bcl-2 was depressed by I/R challenge. Pretreatment with CP at 0.8 g/kg, but not at 0.1 or 0.4 g/kg, upregulated Bcl-2 expression. On the contrary, the expression of Bax was increased remarkably by I/R and downregulated by pretreatment with CP at 0.8 g/kg (Fig. 9, B and D).

**Histology.** Figure 10 presents the result of histological examination of surrounding infarction areas of the left ventricle myocardial tissues in different groups. Compared with the sham group (Fig. 10B), distinct alterations occurred in the surrounding infarction areas of myocardial tissues from the I/R group, including disruption of myocardial fibers, tissue edema, and neutrophil infiltration (Fig. 10C). Of notice, all of the I/R-induced alterations were ameliorated by pretreatment with CP (Fig. 10, D–F), especially at the dose of 0.8 g/kg.

**Changes in myocardial ultrastructure.** The ultrastructures of surrounding infarction areas of the left ventricular myocardial tissues from different groups were examined. Figure 11A presents the representative electron micrographs of capillaries with surrounding tissues. The endothelium of capillaries in the sham group was preserved well, and both the capillary and surrounding tissue displayed normal ultrastructural features (Fig. 11A, A1). The I/R injury led to an apparent swelling of the endothelium with a large number of caveolaes in endothelial cells and prominent edema in the vascular surroundings (Fig. 11A, A2). At 0.4 and 0.8 g/kg of CP, the I/R-evoked endothelial swelling and surrounding tissue edema were conspicuously ameliorated (Fig. 11A, A4 and A5).

The ultrastructure of cardiac muscle cells in each condition was examined as well; the representative electron micrographs are presented in Fig. 11B. In the sham group (B1), cardiac myofibrils stood regularly arranged with well-preserved myofilaments, and mitochondria occupied the cytoplasm between myofibrils with densely packed cristae. The I/R challenge provoked a dramatic injury in cardiac muscle cells (B2), as indicated by disrupted myofilibrils and ruptured mitochondria. Pretreatment with CP at each of the three doses attenuated the ultrastructural alterations induced by I/R (B3, B4, B5).

**IkBα degradation in the myocardium.** To examine the effect of CP on IkBα regulation, Western blot assay was performed for myocardial tissues from different groups. As shown in Fig. 12, I/R induced significant IkBα degradation at 60 min of reperfusion compared with the sham group, pretreatment with CP at 0.4 and 0.8 g/kg significantly inhibited IkBα degradation elicited by I/R challenge, while 0.1 g/kg CP did not affect the level of IkBα in the myocardium following I/R.

**DISCUSSION**

In the present study, we reported for the first time the in vivo data demonstrating that pretreatment with CP significantly attenuates the I/R-induced cardiac impairments, including decreased RBC velocity in coronary microvessels, decreased coronary blood flow, increased expression of CD18 on neutrophils and ICAM-1 on endothelium, albumin leakage across the venular wall, and myocardial injury. Furthermore, the results of TUNEL staining and caspase-3 expression indicate that myocardium injury observed in this study is attributed, at least partly, to apoptosis mediated by Bcl-2 and Bax.

The beneficial effect of CP on microcirculation has been demonstrated in gut I/R-induced hepatic injury (11). However, no study has been reported to address its action on myocardial microcirculatory dysfunction after I/R, despite the fact that it has long been used for the prevention and treatment of I/R-related heart diseases. In the present study, intravital microscopy was applied, enabling visualization in real time of the dynamics of coronary microcirculation in beating hearts. The results are consistent with the findings from the Laser-Doppler Perfusion Imager study, suggesting that pretreatment with CP significantly attenuates the decreases in coronary blood flow, as well as arteriolar/venular flow after I/R. Diminished micro-
vascular flow in the ischemic area after reperfusion is usually referred to as low reflow, a phenomenon resulting from diverse insults where leukocyte recruitment plays a pivotal role (8, 15). The accumulation of leukocytes in microvessels after I/R not only plugs the blood flow, but also causes endothelial injury and barrier dysfunction; both effects contribute to low reflow (24). Thus inhibition of leukocyte adherence to the vascular endothelium exerts protective effects against I/R-induced microcirculatory disorders. For this purpose, monoclonal antibodies against leukocyte adhesion molecules have been tested in clinical trials, although the data are negative in general (21).

While the molecular details underlying the protective effect of CP against I/R-induced microcirculatory disturbance found in this study need to be interrogated in more depth, its inhibitory effect on leukocyte adhesion molecule expression may serve as an important factor in its mechanism of action.

The present study revealed that pretreatment with CP reduces the infarct size and the number of TUNEL-positive cells and improves myocardial structure, pointing to the potential of CP in protecting the myocardium from I/R injury. Myocardium injury after I/R is caused by multiple factors, including oxidative stress, depletion of high energy stores due to hypoxia, and cytotoxic substances released from infiltrated leukocytes, including cytokines, chemotactic factors, and proteinases (1, 7, 19, 30). Considering the fact that SM, one of the major CP ingredients, was demonstrated to have antioxidant ability (20), it is reasonable to propose that CP exerts its protective effect on the myocardium through mechanisms involving suppressed ROS production, which may, in turn, reduce leukocyte adhesion and infiltration and thus improve oxygen supply to the cardiac muscle cells. This notion is further supported by the data that the I/R injury-induced MDA production, an indicator of lipid peroxidation, was attenuated by administration of CP.

It is well recognized that necrosis and apoptosis are two major types of cell death in tissues subject to I/R (29). This study does not discriminate the relative contribution of each type to infarct size in the myocardium. Nevertheless, I/R-induced apoptosis of cardiac myocytes was attenuated by pretreatment with CP, as evidenced by the decrease in the number of TUNEL-positive cells and caspase-3 expression. Furthermore, the increase of Bcl-2 and decrease of Bax indicates that the intrinsic apoptotic pathway is at least one of the targets that CP acts on (5). More studies that determine the activity of caspase-8 are required to clarify whether or not the extrinsic apoptotic pathway is involved in this process.
I/R stimulates the production of peroxides that induce the degradation of IkB, and then activation of NF-κB, resulting in expression of various adhesion molecules and apoptosis-related proteins (14, 35). Studies using a model of myocardial I/R injury have shown that blockade of NF-κB reduces infarct size and protects myocytes from ischemic insult (17). Water-soluble fraction from SM was reported to inhibit the TNF-α induced translocation of NF-κB from the cytoplasm to the nucleus on human umbilical vein endothelial cells (4). Our results agree with previous studies that the IkB was degradation induced by myocardial I/R and further propose that CP results agree with previous studies that the IκB nucleus on human umbilical vein endothelial cells (4). Our results agree with previous studies that the IκB was degradation induced by myocardial I/R and further propose that CP inhibits IkB degradation. Nonetheless, the underlying mechanism needs to be further elucidated.

In China, CP is currently used for treatment of angina in the clinic by administration for 4 wk at a dose of 0.75 g kg−1 day−1. In the present study, the doses of CP used were 0.1, 0.4, and 0.8 g/kg, which were designed to be 1.2-, 4.9-, and 9.9-fold higher, respectively, than the equivalent effective dose for treatment of human angina. The results of the present study revealed that administration of CP at 0.1 and 0.4 g/kg may attenuate the increase in MDA level and the number of TUNEL-positive cells in rat myocardium, alleviating morphological disorder in myocardial tissue induced by I/R, while administration of CP at a dose of 0.8 g/kg attenuates I/R-elicited rat myocardium injury and cardiac microcirculation disturbance as well. No report is available as yet regarding whether or not pretreatment with CP by a single dose may attenuate I/R-induced cardiac microcirculation disturbance and myocardial tissue injury, and, if yes, what is the effective dose. Nonetheless, the present result suggests that, if CP is intended for use of preventing I/R-induced cardiac microcirculation disturbance and myocardial tissue injury, the dose should be higher than that for treatment of angina.

In summary, pretreatment with CP (0.8 g/kg) attenuates I/R-induced coronary microcirculatory disturbance and albumin leakage, which may be attributed to its inhibitory effect on CD18 and ICAM-1 expression. Furthermore, CP may protect the myocardium from damage following I/R injury by suppressing the intrinsic apoptotic pathway. The protective effects of CP on rat coronary microcirculatory disturbance and myocardial damage may be related to inhibiting degradation of IkBα.

**GRANTS**

This work was supported, in part, by the International Science and Technology Cooperation Project from the Ministry of Science and Technology of the People’s Republic of China (2006DFA32340).

**DISCLOSURES**

No conflicts of interest are declared by the author(s).

**REFERENCES**


21. Penuthasv SV, Koneru S, Zhan L, John S, Monen VP, Prasad K, Maulik N. Secoisolariciresinol diglycoside induces neovascularization-


