

Total flavonoid extract from *Coreopsis tinctoria* Nutt. protects rats against myocardial ischemia/reperfusion injury

Ya Zhang¹, Changsheng Yuan¹, He Fang¹, Jia Li¹, Shanshan Su¹, Wen Chen^{2*}

¹ Ministry of Education, Pharmacy Shihezi University Xinjiang China

² Pharmacy Shihezi University Xinjiang China

ARTICLE INFO

Article type:

Original article

Article history:

Received: Oct 31, 2015

Accepted: Mar 3, 2016

Keywords:

Anti-apoptosis

Anti-oxidant

Anti-inflammatory

Cardio-protective

Coreopsis tinctoria Nutt

ABSTRACT

Objective(s): This study aimed to evaluate the protective effects of total flavonoid extract from *Coreopsis tinctoria* Nutt. (CTF) against myocardial ischemia/reperfusion injury (MIRI) using an isolated Langendorff rat heart model.

Materials and Methods: Left ventricular developed pressure (LVDP) and the maximum rate of rise and fall of LV pressure ($\pm dp/dt_{max}$) were recorded. Cardiac injury was assessed by analyzing lactate dehydrogenase (LDH) and creatine kinase (CK) released in the coronary effluent. Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) levels were determined. Myocardial inflammation was assessed by monitoring tumor necrosis factor-alpha (TNF- α), C-reactive protein (CRP), interleukin-8 (IL-8), and interleukin-6 (IL-6) levels. Myocardial infarct size was estimated. Cell morphology was assessed by 2,3,5-triphenyltetrazolium chloride and hematoxylin and eosin (HE) staining. Cardiomyocyte apoptosis was determined by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining.

Results: Pretreatment with CTF significantly increased the heart rate and increased LVDP, as well as SOD and GSH-Px levels. In addition, CTF pretreatment decreased the TUNEL-positive cell ratio, infarct size, and levels of CK, LDH, MDA, TNF- α , CRP, IL-6, and IL-8.

Conclusion: These results suggest that CTF exerts cardio-protective effects against MIRI via anti-oxidant, anti-inflammatory, and anti-apoptotic activities.

► Please cite this article as:

Zhang Y, Yuan Ch, Fang H, Li J, Su Sh, Chen W. Total flavonoid extract from *Coreopsis tinctoria* Nutt. protects rats against myocardial ischemia/reperfusion injury Iran J Basic Med Sci 2016; 19:1016-1023.

Introduction

Acute myocardial ischemia is a major cause of mortality and morbidity worldwide (1). Although timely reperfusion is crucial for rescuing dying myocardial tissue, restoration of circulation can increase the severity of reperfusion injury symptoms, including cardiomyocyte dysfunction and cell death in a phenomenon known as myocardial ischemia/reperfusion injury (MIRI)(2-3). Therefore, prevention and management of MIRI is a crucial concern in coronary heart disease surgery. Previous studies have shown that calcium overload, energy metabolism disorders, excessive reactive oxygen species (ROS) abundance, inflammatory reactions, and apoptosis are involved in the pathogenesis of MIRI (4).

Flavonoids are a vast group of polyphenols found ubiquitously in vegetables and fruits (5). Some flavonoids might be useful as chemopreventive agents for cardiovascular diseases (6). Population studies have shown that flavonoid intake in the human diet is inversely correlated with mortality

from cardiovascular diseases (7). Epidemiological, *in vitro*, and animal studies have indicated that flavonoids have beneficial impacts on parameters associated with cardiovascular disease, including lipoprotein oxidation, blood platelet aggregation, and vascular reactivity (8). The cardio-protective effect of flavonoids can be attributed to their anti-oxidant, anti-thrombogenic, anti-inflammatory, and anti-apoptotic properties, while increased flavonoid intake is thought to play a key role in reducing the risk of developing cardiovascular diseases (9). Luteolin has been used experimentally and in clinical practice to protect myocardial tissue from MIRI (10). whereas epigallocatechin-3-gallate, the major flavonoid in green tea, has been shown to attenuate MIRI in several animal species (11).

Coreopsis tinctoria Nutt. is an annual herbaceous plant belonging to the Asteraceae family. *C. tinctoria* is native to North America, but has spread worldwide, especially to the southern part of the Xinjiang Uygur Autonomous Region in China. *C. tinctoria* is used in the management of diabetes

*Corresponding author: Wen Chen. Pharmacy Shihezi University Xinjiang China; Tel: +8609932055002; Fax: +8609932055002; email: chen-wen2000@126.com

(12), as well as for its vasorelaxant (13), anti-inflammatory (14), and anti-oxidant activities (15). Previous studies have also revealed that *C. tinctoria* has a particularly high content of flavonoids (16). In this study, we evaluated the cardio-protective effects of the total flavonoid extract from the flower of *C. tinctoria* and investigated the mechanisms underlying these effects.

Materials and Methods

Sample preparation

The flower buds of *C. tinctoria* were collected in August of 2014 in Pishan country (Hetian region, Xinjiang, China). Botanist Peng Li Shihizi University (Xinjiang, China) confirmed the authenticity of the material. We extracted total flavonoids from these flowers (CTF) in our laboratory by a previously reported method (17). In brief, the dried and powdered flower buds of *C. tinctoria* (2.0 kg) were consecutively extracted twice under reflux with 95% ethanol, after which the solvent was removed by evaporation to yield an ethanol extract. The ethanol extract was purified using polyamide resin to obtain a 70% ethanol extract eluate, which was dried over anhydrous magnesium sulfate, after which the solvent was removed under vacuum (40 °C) and the sample was lyophilized. The extract was dissolved in 70% ethanol solution to allow analysis of total flavonoid content using a colorimetric-based method assay (18). Briefly, CTF (45.5 mg) was placed in a 100-ml volumetric flask with 70% ethanol solution. The CTF solution (1.0 ml) was mixed with 4.0 ml 70% aqueous ethanol, after which 0.3 ml NaNO₂ (5%, w/v) was added. After 6 min, 0.3 ml AlCl₃ (10%, w/v) and 2.0 ml NaOH (1 M) were added, followed by the addition of distilled water to reach a volume of 10.0 ml. The resulting solution was mixed and incubated for 15 min at room temperature. The experimental and control solutions were scanned by a UV-2600 spectrophotometer using quartz cuvettes (1.0 cm) (Shimadzu, Japan) at 510 nm. The control solution contained all reaction reagents except for the test sample. The standard curve regression equation was as follows: $A = 0.010235C + 0.0119$ ($R^2 = 0.9993$) (where A is the absorption and C is the rutin concentration in µg/ml). The flavonoid content of each solution was calculated from the calibration curve and expressed as mg rutin equivalents (RE) per gram dry weight (DW) of extract (mg RE/g DW). The total flavonoid content of CTF was 828.5 ± 3.6 mg RE/g DW.

Test compounds, chemicals, and reagents

2,3,5-Triphenyltetrazolium chloride (TTC) was purchased from Sigma Chemical Co (St Louis, MO, USA). All other reagents used in this study were purchased from commercial suppliers and were of analytical grade.

Experimental animals and treatment

All experimental procedures were approved by the Institutional Animal Care and Use Committee of the National Institute of Pharmaceutical Education and Research.

Sprague–Dawley male rats (250–300 g each) were supplied by the Xinjiang Medicine University Medical Laboratory Animal Center (License Number: SCXK(xin)2011-0003). The rats were maintained under natural light (14 hr) and dark (10 hr) conditions in a facility maintained at 25 ± 2 °C. The rats were fed a standard laboratory diet and tap water ad libitum during the experimental period.

The rats were randomly divided into five experimental groups: control (sham), ischemia/reperfusion (I/R), and three total flavonoid treatment groups. For the control group, the hearts were perfused throughout 95 min stabilization. For the I/R group, the hearts were exposed to 20 min of zero-low global ischemia and 45 min of reperfusion after 30 min of stabilization. For the treatment groups, the hearts were stabilized for 20 min and exposed to Krebs–Henseleit (K-H) buffer (118 mM NaCl, 1.2 mM KH₂PO₄, 4.7 mM KCl, 1.7 mM CaCl₂, 1.2 mM MgSO₄, 20 mM sodium acetate, and 10 mM glucose (pH 7.4)) containing CTF (5, 10, or 20 µg/ml per rat) for 10 min. Finally, the hearts were subjected to global ischemia for 20 min and reperfusion for 45 min (Figure 1).

Langendorff isolated perfused heart preparation

The rats were anesthetized via intraperitoneal injection of chloral hydrate (0.35 g/kg). Next, heparin sodium (250 U/kg), an anticoagulant, was administered intraperitoneally to each rat, after which a thoracotomy was conducted to remove the heart. Each heart was immediately placed into ice-cold K-H buffer (19). The excised hearts were cannulated through the aorta on a

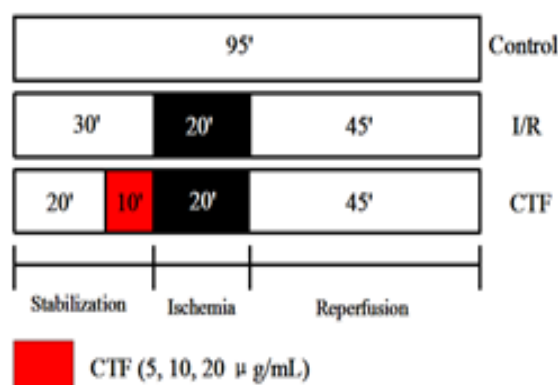


Figure 1. Heart ischemia/reperfusion protocol and groups.

Langendorff apparatus and perfused with K-H buffer that was bubbled with 95% O₂ and 5% CO₂ at 37 °C throughout the experiment. The perfusion was initiated at 75 mmHg. A fluid-filled latex balloon combined with a Gulton–Statham pressure transducer (Ametek, Berwyn, PA, USA) was introduced into the left ventricle through the left auricle to record pressure.

Monitoring of heart hemodynamic parameters

Hemodynamic parameters were constantly monitored with a computer-based data collection system (PC PowerLab with Chart 5 software, 4S AD Instruments, New South Wales, Australia). During the experiments, a 4S AD Instruments Biology Polygraph (Powerlab, New South Wales, Australia) was used to measure the following cardiac functional parameters: left ventricular end-diastolic pressure (LVEDP), left ventricular systolic pressure (LVSP), left ventricular developed pressure (LVDP; LVDP =

LVSP - LVEDP), heart rate (HR), and the maximum rise/fall velocity of the left intra ventricular pressure ($\pm dp/dt_{max}$). Coronary effluents were collected at 1 min intervals at chosen time points to determine coronary flow (CF).

Assessment of myocardial damage

Myocardial damage was evaluated by measuring the activity of lactate dehydrogenase (LDH) and creatine kinase (CK) released into the coronary effluent (20). Samples were collected from the coronary effluent prior to the 20-min ischemia period and after 20 and 45 min of reperfusion. LDH and CK kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) were used to spectrophotometrically assay levels of LDH and CK.

Evaluation of myocardial infarct size

After reperfusion, the hearts were removed, flushed

Table 1. Effect of flavonoid extract from *Coreopsis tinctoria* Nutt. (CTF) on cardiac function in rats subjected to I/R

Physical index	Reperfusion (%)		
	15 min	30 min	45 min
LVDP			
control	95.06±1.49	93.30±1.61	92.24±2.23
I/R	41.27±1.67##	47.78±1.93##	49.16±1.94 ##
5 µg/ml CTF	63.03±4.34 **	58.16±6.10 **	57.43±4.23 **
10 µg/ml CTF	75.87±3.11 **	72.93±4.44 **	71.57±2.69 **
20 µg/ml CTF	84.40±2.27 **	82.36±3.92 **	81.49±3.98 **
+dp/dt_{max}			
Control	103.95±7.77	102.14±8.51	103.50±7.05
I/R	43.38±3.30##	50.89±2.88##	52.11±5.66##
5 µg/ml CTF	56.88±4.69 **	55.96±4.29*	52.43±4.49
10 µg/ml CTF	68.99±4.11 **	72.37±4.35 **	69.48±4.78**
20 µg/ml CTF	87.39±6.87**	87.39±6.87**	86.16±7.08**
-dp/dt_{max}			
Control	97.62±3.62	97.12±3.90	96.33±3.97
I/R	47.83±3.94 ##	53.74±5.16 ##	52.63±5.41 ##
5 µg/ml CTF	53.49±5.24 *	56.85±4.00	53.73±3.25
10 µg/ml CTF	62.23±2.95 **	63.75±2.32 **	61.62±3.43 *
20 µg/ml CTF	83.18±3.48 **	83.43±2.18**	83.38±2.51 **
CF			
control	100.99±4.26	99.50±4.07	100.34±2.90
I/R	89.54±5.80 #	89.42±6.34 #	89.11±6.51 #
5 µg/ml CTF	91.87±6.45	91.19±6.42	91.64±7.21
10 µg/ml CTF	94.90±4.42	95.69±4.80 *	96.49±5.21 *
20 µg/ml CTF	94.71±4.48	94.36±4.43	94.97±4.60
HR			
Control	94.54±2.85	95.70±3.23	96.57±3.85
I/R	73.56±8.33 ##	70.18±4.75 ##	64.65±5.76 ##
5 µg/ml CTF	76.65±4.64	73.28±4.43	67.91±3.65
10 µg/ml CTF	81.33±5.47*	76.03±4.19 *	69.37±2.67
20 µg/ml CTF	83.60±7.82 *	80.15±7.72**	78.17±8.14**

(All values are presented as mean±SD; n = 8). Left ventricular developed pressure (LVDP); maximum rate of rise (+dp/dt_{max}) and fall (-dp/dt_{max}) of LV pressure; coronary flow (CF); heart rate (HR). ##P<0.01 vs. control; *P<0.05; **P<0.01 vs. I/R

with phosphate-buffered saline (PBS), placed at -20 °C for 15 min, and cut into five 1-mm-thick slices. All sections were incubated for 15 min in 1% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma Co., St Louis, MO, USA) at 37 °C in the dark, fixed in 10% formaldehyde solution, and photographed with a digital camera to distinguish red-stained normal tissue and the white-unstained infarcted tissue. The infarct zone was measured and analyzed by Image-Pro Plus image analysis software (Version 4.1, Media Cybernetics, LP, Silver Spring, MD, USA). The infarction size percentage was calculated as the ratio of the infarct volume to the total volume of the slices.

Measurements of anti-oxidant indices

After perfusion treatments, the hearts were collected and stored at -70 °C. The frozen tissue was ground into a powder using a liquid nitrogen-chilled tissue grinder, weighed, and homogenized in the appropriate buffer for each tissue analysis. Superoxide dismutase (SOD) activity, glutathione peroxidase (GSH-Px) activity, and malondialdehyde (MDA) levels were used as indices of reactive oxygen species (ROS) and membrane lipid peroxidation levels. SOD activity, GSH-Px activity, and MDA levels were measured using commercial kits as per the manufacturer's instructions (JianCheng Bioengineering Institute, Nanjing, China).

Inflammation assay

TNF- α , IL-6, IL-8, and CRP levels were analyzed spectrophotometrically according to the instructions provided with the rat tumor necrosis factor alpha (TNF- α) ELISA kit, rat interleukin 6 ELISA kit, rat interleukin 8 ELISA kit, and rat C-reactive protein ELISA kit (Tsz Biosciences, Boston, MA, USA).

General histology

The heart of each rat was fixed in 10% buffered formalin and preserved at normal temperature, after which it was subjected to HE staining and viewed under an optical microscope. A small piece (2 mm \times 1 mm \times 1 mm) of subendocardial myocardium from the root of the left ventricular papillary muscle was obtained and fixed in 0.1 mM/l phosphate buffer (pH 7.2), which included 3% glutaraldehyde and 1.5% paraformaldehyde, at 4 °C. The piece was cut into small pieces of 1 mm³ and fixed in the solution mentioned above for 4 h. Next, the piece was rinsed with phosphate buffer solution and fixed in 1% osmic acid at 4 °C for 1.5 h, after which the tissue was dehydrated using alcohol followed by dimethylbenzene and embedded in epoxy resin 618. The tissue was located by semi-thin sectioning and sliced into ultra-thin sections (60 nm in thickness). The sections were dyed with uranium acetate and lead citrate and observed under an optical microscope.

Determination of myocardial apoptosis

At the end of the protocols, the hearts were sectioned and fixed in neutral formalin (10% formaldehyde in PBS, pH 7.4). TUNEL was conducted using an *in situ* Cell Death Detection Kit, POD (Roche, Mannheim, Germany) according to the manufacturer's instructions. The slides were analyzed under an optical microscope. For quantitative analysis, eight randomly chosen areas of TUNEL-stained slices were counted. The TUNEL index (%) was calculated as the ratio of the number of TUNEL-positive cells to the total number of cells.

Statistical analysis

All data were expressed as mean \pm SD. The data were analyzed using one-way ANOVA, followed by Student's *t*-test. In all cases, values of $P < 0.05$ were considered statistically significant. Statistical analysis was performed using SPSS (IBM Co. Armonk, NY, USA).

Results

CTF produces recovery of I/R-induced cardiac function

The hemodynamic parameters of all groups in response to reperfusion are summarized in Table 1. In comparison with the control group, the I/R group showed less recovery of cardiac function, whereas the CTF treatment (5, 10, and 20 μ g/ml) groups showed significantly improved function in comparison with that of the I/R group. Hemodynamic data showed that CTF treatment rescued cardiac dysfunction after I/R.

Effects of CTF on LDH and CK Activities in the Coronary Effluent

The activities of myocardial-specific enzymes LDH and CK in the coronary effluent were used as markers of myocardial injury. Prior to ischemia, the activities of LDH and CK in the coronary effluent of all groups were similar (Table 2). However, after 20 min of ischemia, followed by 20 and 45 min of reperfusion, release of LDH and CK into the coronary effluent was markedly increased in the I/R group in comparison with that of the control group. Pretreatment with 20 μ g/ml CTF significantly reduced the I/R-induced increases in LDH and CK release in the rat heart ($P < 0.01$).

CTF limits the size of the I/R-induced infarct

The increase in the size of the I/R-induced infarct is the most obvious indicator of MIRI. As shown in Figure 2B, the hearts subjected to global myocardial ischemia for 20 min followed by 45 min of reperfusion showed a significantly increased infarct area (65.23 \pm 10.13%) in comparison with that of the I/R group, whereas pretreatment with 10 and 20 μ g/ml CTF significantly reduced the I/R-induced

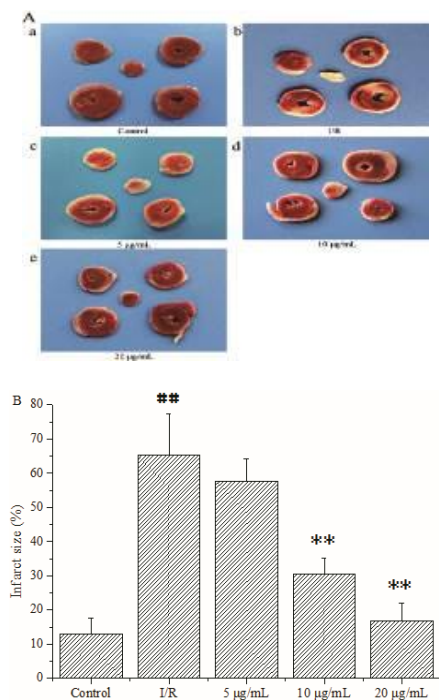


Figure 2. Effect of CTF on infarct size in rats subjected to I/R. A: Representative heart sections at various levels after staining with 1% 2,3,5-triphenyltetrazolium chloride (TTC) to identify infarct tissue (white) and normal muscle tissue (orange brown). a: Control; b: I/R; c: 5 µg/ml CTF; d: 10 µg/ml CTF; e: 20 µg/ml CTF. B: Summarized data are presented as mean±SD (n=8, each) ###P<0.01 vs. control; *P<0.05; **P<0.01 vs. I/R. CTF: *Coreopsis tinctoria* Nutt. flower total flavonoids

infarct size to 30.6±4.61% and 16.8 ±6.43%, respectively, of that of the control rats (P<0.01).

Effect of CTF on I/R-induced oxidative stress in the myocardium

Oxidative stress plays an important role in the progression of ischemic heart injury. SOD, GSH-Px, and MDA are indicators of oxidation. To identify the cardioprotective mechanism of CTF, the effects of CTF on SOD activity, GSH-Px activity, and MDA production in

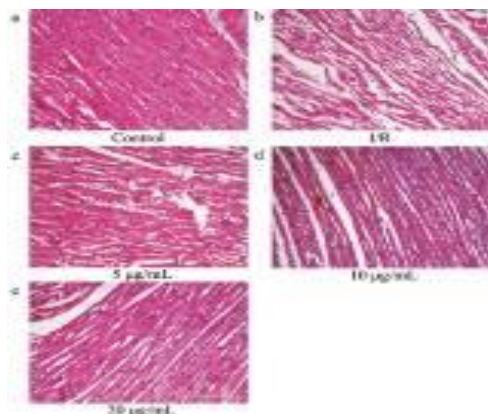


Figure 4. Effects of CTF on cell morphology and hematoxylin and eosin (HE) staining (×200) (n = 8). a: Control; b: I/R; c: 5 µg/ml CTF; d: 10 µg/ml CTF; e: 20 µg/ml CTF. CTF: *Coreopsis tinctoria* Nutt. flower total flavonoids

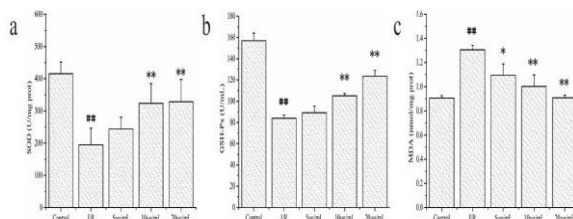


Figure 3. Effects of CTF on SOD (a), GSH-Px (b), and MDA (c) in rats subjected to I/R (all values are presented as mean ± SD; n = 8). ##P<0.01 vs. control; *P<0.05; **P<0.01 vs. I/R. CTF: *Coreopsis tinctoria* Nutt. flower total flavonoids; GSH-Px: Glutathione peroxidase; SOD: Superoxide dismutase; MDA: malondialdehyde

myocardial tissue in response to I/R injury were investigated. As shown in Figure 3, the SOD and GSH-Px activity levels of the groups treated with 10 and 20 µg/ml CTF were increased significantly (both P<0.01) in comparison with those of the I/R group, whereas these CTF-treated groups showed significantly reduced MDA levels (P<0.01). The group pretreated with 5 µg/ml CTF showed no significant differences in SOD activity, GSH-Px activity, and MDA production in comparison with the I/R group.

Effect of CTF on myocardial morphology

Changes in the morphology of myocardial tissue were assessed by HE staining. Optical micrographs of the myocardial structure are shown in Figure 4. Myocardial tissue from the control rats showed normal morphology; cardiomyocytes were arranged tightly and orderly, the muscle membrane was not damaged, there was no edema between cells, and muscle fibers showed no fracture, degeneration, or necrosis. In contrast, the myocardial structure of the I/R group (Figure 4b) showed loosely and irregularly arranged muscle fibers, severe edema between cells, pyknotic nuclei, many infiltrated inflammatory cells, and fracture, degeneration, and necrosis of muscle fibers. As shown in Figure 4d and Figure 4e, pretreatment with 10 and 20 µg/ml CTF significantly reduced MIRI; however, 5 µg/ml CTF did not affect MIRI (Figure 4c).

CTF reduces cardiomyocyte apoptosis

Accumulating evidence indicates that cell loss through apoptosis is the predominant mode of post-ischemic cardiomyocyte death in the heart and contributes significantly to impairment of cardiac performance, suggesting that reducing cardiomyocyte loss through inhibition of cell death is a reasonable approach to protecting the myocardium (21-23). Therefore, TUNEL staining was performed to observe cardiomyocyte apoptosis. The apoptosis index of the I/R group was significantly higher than that of the control group (P<0.01), whereas the groups pretreated with at 10 µg/ml (Figure 5d) and 20 µg/ml (Figure 5e) CTF showed a clearly reduced number of apoptotic cells. The apoptosis rate of the group pretreated with 5 µg/ml CTF was not significantly different from that of the I/R group (Figure 5c).

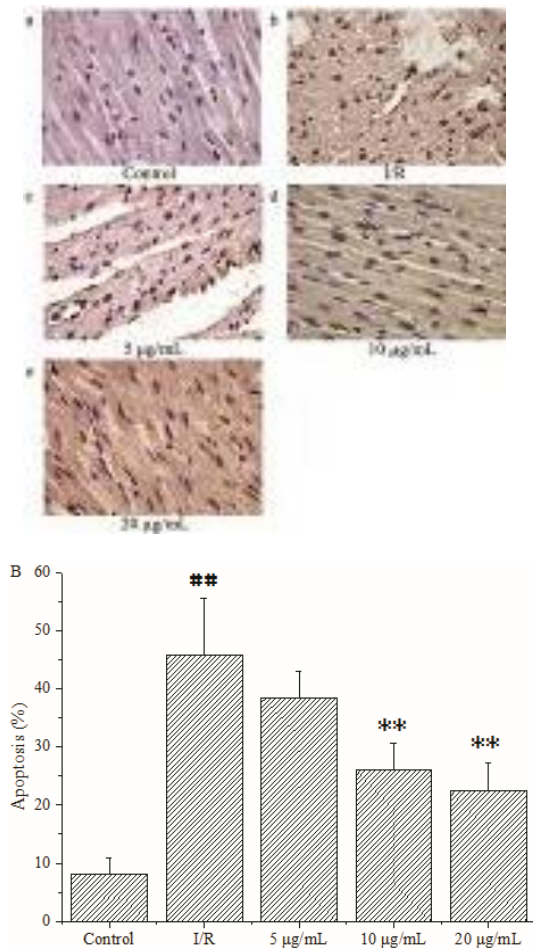


Figure 5. Effects of CTF on cardiomyocyte apoptosis in rats subjected to I/R ($\times 400$)
 A: Representative heart sections at various levels after staining with TUNEL for TUNEL-positive cells (brown) and normal cells (blue). a: Control; b: I/R; c: 5 $\mu\text{g/ml}$ CTF; d: 10 $\mu\text{g/ml}$ CTF; e: 20 $\mu\text{g/ml}$ CTF
 B: Summarized data are presented as mean \pm SD (n = 8, each)
 ## $P < 0.01$ vs. control; * $P < 0.05$; ** $P < 0.01$ vs. I/R

CTF reduces I/R-induced inflammation

Recent studies have shown that levels of inflammatory cytokines are directly related to the extent of MIRI and the number of necrotic cells. The possible mechanisms underlying the cardio-protective activity of CTF were identified by measuring levels of inflammatory cytokines (TNF- α , C-reactive protein (CRP), interleukin-8 (IL-8), and interleukin-6 (IL-6)) following I/R. The IL-6 levels of the groups pretreated with 10 $\mu\text{g/ml}$ (721.2 \pm 90.3 pg/ml) and 20 $\mu\text{g/ml}$ CTF (628.7 \pm 81.1 pg/ml) were significantly lower (both $P < 0.01$) than that of the I/R group (1077.8 \pm 124.4 pg/ml; Figure 6a). The TNF- α levels of the groups pretreated with 10 $\mu\text{g/ml}$ (616.3 \pm 59.0 pg/ml) and 20 $\mu\text{g/ml}$ CTF (559.3 \pm 58.2 pg/ml) were significantly lower than that of the I/R group (762.9 \pm 39.1 pg/ml) (both $P < 0.01$; Figure 6b). The IL-8 level of the control group was 361.8 \pm 71.3 pg/ml, whereas that of the I/R group was

1025.4 \pm 42.2 pg/ml (Figure 6c). In contrast, the IL-8 levels of the groups treated with 10 and 20 $\mu\text{g/ml}$ CTF (784.4 \pm 64.1 and 600.9 \pm 35.6 pg/ml, respectively) were significantly decreased in comparison with that of the I/R group (both $P < 0.01$). The CRP levels of the groups treated with 10 and 20 $\mu\text{g/ml}$ CTF (528.9 \pm 30.6 and 498.5 \pm 31.7 pg/ml, respectively) were decreased significantly in comparison with that of the I/R group (622.8 \pm 55.7 pg/ml) (both $P < 0.01$; Figure 6d). The levels of IL-6 and IL-8 in the group treated with 5 $\mu\text{g/ml}$ CTF were decreased significantly in comparison with those of the I/R group ($P < 0.05$), but the levels of TNF- α and CRP in these groups were not significantly different.

Discussion

Our study revealed for the first time that the total flavonoids of the flowers of *C. tinctoria* produce cardio-protective effects by suppressing myocardial injury in a global I/R model. CTF reduced myocardial enzyme leakage, decreased I/R-induced cardiomyocyte apoptosis, and enhanced anti-oxidant defense following I/R injury in rats. Thus, the cardio-protective effects of CTF may be attributed to its anti-oxidant, anti-apoptotic, and anti-inflammatory activities.

ROS are one of the main contributors to cell damage after ischemia/reperfusion (24-26). DNA, proteins and lipids can be oxidized by ROS, thereby causing hypofunction or dysfunction of these molecules (27). ROS-induced damage of enzymes involved in cellular protein repair or removal may aggravate the pernicious effect of ROS (28). Previous studies have shown that, under normal conditions, tissues can maintain the balance between generation and clearance of ROS, but I/R disrupts this balance. Therefore, activation of the endogenous defense system, particularly the anti-oxidant enzyme system (e.g., SOD and GSH-Px), is a useful approach to limiting oxidative stress-induced tissue damage (29). MDA produced by lipid peroxidation results in release of myocardial enzymes and destruction of structural proteins and cellular structures (30). The present study showed that SOD and GSH-Px activities were significantly increased by CTF, whereas MDA levels were significantly decreased. Perfusate CK and LDH levels in the CTF treatment groups, particularly in the 20 $\mu\text{g/ml}$ group, were significantly lower than those of the I/R group. Therefore, CTF can act as a myocardial protectant via its anti-oxidant effects.

Changes in cardiac function are closely related to the degree of MIRI. The function of the heart mainly depends on the contraction and relaxation properties of the ventricular muscle (31). The present study showed significant myocardial dysfunction, including marked reduction of LVSP and $+dp/dt_{max}$, after global ischemia and reperfusion, whereas the CTF treatment groups showed significant improvement in cardiac diastolic

dysfunction. Moreover, the size of the myocardial infarction area is directly related to the prognosis of the patient; protection of the myocardium results in reduction of the infarction area and an improved prognosis. Measurement of the infarction area is considered to be a crucial criteria for evaluating MIRI (32). We have shown that CTF markedly reduced the infarction area size. Furthermore, recent studies have shown that apoptosis plays a crucial role in various harmful stimuli, especially persistent I/R. Therefore, inhibition of cardiomyocyte apoptosis is an important approach to preventing myocardial I/R injury (33). CTF markedly reduced the cardiomyocyte apoptosis rate in our study.

Previous studies have confirmed that inflammation is present throughout I/R-injured myocardial tissue. I/R can induce monocytes, macrophages, and neutrophils to release TNF- α , IL-6, and other inflammatory cytokines, which enhance inflammatory reactions and lead to myocardial injury (34). Many inflammatory cytokines, such as TNF- α , IL-8, CRP, and IL-6, play important roles in inflammatory reactions and are closely related to the development and progression of cardiovascular disease (35). Among these cytokines, TNF- α is a primary cytokine, which induces the release of "messenger" cytokines that increase levels of IL-6, CRP, and other acute reactants (36). This action results in enhanced adhesion of leukocytes to endothelial cells, aggravated microvascular occlusion in the ischemic zone, and cellular damage, resulting in further cardiomyocyte injury (37). To study the relationship between the anti-inflammatory effects and cardio-protective properties of CTF, we performed an experiment to analyze whether CTF influences the changes in IL-6, IL-8, TNF- α , and CRP levels induced by I/R. In our study, inflammatory factors IL-8, IL-6, CRP, and TNF- α were released into the hearts of the I/R group, but CTF significantly repressed this inflammatory cascade. Therefore, suppression of inflammatory cytokine infiltration by CTF treatment may underlie the cardio-protective effects of CTF after reperfusion. These mechanisms should be further investigated in future studies.

Conclusion

Our data demonstrate the protective effect of CTF against I/R injury and strongly suggest that the mechanisms underlying this cardio-protective effect of CTF involve its anti-oxidant, anti-apoptotic, and anti-inflammatory properties. Our findings suggest that CTF may be an effective therapeutic agent against MIRI that could be used in the clinic.

Acknowledgment

This study was supported by Construction of innovative drugs based on natural resources in Xinjiang incubator base (2011ZX0940-007) to Wen Chen.

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