EXPERIMENTAL STUDY

Crocin Attenuates Oxidative Stress and Myocardial Infarction Injury in Rats

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Summary

Oxidative stress and excessive nitric oxide (NO) production play considerable roles in infarction-induced injury impairing cardiac functions. Crocin is the active constituent of *Crocus sativus* (saffron) with antioxidant properties and has protective effects against disturbances induced by ischemia in many organs. The present study aimed to investigate the protective effects and the underlying mechanisms of crocin on myocardial infarction (MI)-induced injury in rats *in vivo*. MI rats were intraperitoneally injected with crocin at different doses for seven successive days after coronary ligation. Infarct size, hemodynamic studies, and capillary density were evaluated. Levels of oxidative stress, inducible nitric oxide synthase (iNOS), endothelial nitric oxide synthase (eNOS), and their corresponding phosphorylation expressions were then measured. Crocin decreased infarct size, left ventricular (LV) end-diastolic pressure, and LV minimum dP/dt while increased LV maximum dP/dt and percentage of LV fractional shortening dose dependently. Capillary density was markedly increased after crocin treatment. Crocin enhanced superoxide dismutase activity and reduced malondialdehyde levels as well as inhibited excessive production of NO through downregulating iNOS as well as upregulating eNOS during MI-induced injury. This study reveals that crocin improves MI-induced impairments in cardiac function, which is associated with its antioxidant properties.

Key words: Infarct size, Capillary density, Anti-oxidant, Nitric oxide

Myocardial infarction (MI), one of the most common types of ischemic heart diseases, remains to be a leading cause of morbidity and mortality worldwide. Patients who survive the acute MI stage always face an enhanced risk of post-MI left ventricular (LV) remodeling and heart failure. Adverse ventricular remodeling has been reported to be associated with ventricular dysfunction and contributes to poor outcome of MI. Oxidative stress and excessive nitric oxide (NO) production play critical roles in MI-induce injury. Once MI occurs, the production of reactive oxygen species (ROS) at the infarction site quickly increases, which then induces cardiomyocyte apoptosis and structural damage. A recent research shows that the level of oxidative stress, as one of the metabolic features, began to change since early MI in a rat model. Another interesting study indicates that oxidative stress in the rostral ventrolateral medulla results in obvious sympathoexcitation in a rat model of heart failure. In living cells, both enzymatic and nonenzymatic defense mechanisms exist to balance various oxidative challenges encountered, and drugs have been reported to elicit cardioprotective effects through upregulating activities of enzymic antioxidants in MI model.

Crocin, the active constituent of *Crocus sativus* (saffron), has been shown to exert various health benefits and is universally regarded as a phytotherapeutical drug with few or no side effects. Crocin is reported to exert antioxidant properties that have protective effects against disturbances induced by ischemia/reperfusion in the brain, kidney, and skeletal muscles. So far the effects of crocin on MI-induced injury *in vivo* and the underlying mechanisms have not been well reported.

In this study, MI was induced by coronary ligation in rat heart, followed by treatment with crocin at different doses, to investigate its roles in MI-induced injury and elucidate the potential mechanisms *in vivo*. The present study aimed to provide theoretical evidences for the clinical application of crocin in treating MI.

Methods

Animals: Adult male Wistar rats (230-260 g) with an average age of 2-3 months were purchased from SLAC (Shanghai, China). Rats were maintained in a 12-hour light-dark cycle. All research and animal care procedures were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Tianjin First Center Hospital.

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were approved by the Ethics Committee of Tianjin First Center Hospital for Animal Research.

**Grouping and drug treatment:** The rats were randomly divided into the 0, 20, 40, 60 crocin groups, with 10 rats in each group. After the model was successfully established, the rats in the 20, 40, and 60 crocin groups were intraperitoneally injected 20, 40, and 60 mg/kg of crocin (dissolved in PBS; Sigma-Aldrich, St. Louis, MO, USA), respectively, for seven successive days. The dose chosen in this study was based on our pilot study, which indicated that dose lower than 20 mg/kg was not efficient to protect against the MI-induced impairments (data not shown). The rats in the 0 crocin group were injected PBS as the vehicle control. In the present study, the doses of crocin were designed according to previously described.[10,11]

**Establishments of myocardial infarction models:** The left coronary artery was ligated to induce myocardial infarction, as described previously,[12] with minor modifications. Briefly, rats were anesthetized by pentobarbital sodium (30 mg/kg, i.p.), followed by artificial ventilation using a rodent respirator. After the left thoracotomy, the heart was exposed and the left coronary artery was ligated 2-3 mm from its origin between the pulmonary artery conus and the left atrial appendage with a 6-0 prolene suture. After coronary ligation, the LV anterior wall presented a pale appearance as a result of the lack of blood supply and pulsed weakly. The ECG showed ST segment and Q wave changes, indicating that MI models were successfully established. The chest was closed until a stable cycle was obtained.

**Masson’s trichrome staining:** Rats were sacrificed 4 weeks after MI surgery. The LV myocardium was dissected, fixed in 10% formalin, embedded in paraffin, and sectioned into 10-μm-thick slices transversely. Then after deparaffinization slides were stained with a Masson’s trichrome staining kit (Sigma-Aldrich) to detect fibrosis in the myocardium according to the manufacturer’s instructions.

**Determination of the myocardium infarct size:** Infarct size was determined by analyzing images stained with the Masson’s trichrome using Microsoft Image Composite Editor as previously described.[13] As Masson’s trichrome stained cardiac muscle tissue red and fibrosis blue, blue regions of the image were identified and used to determine infarct size. The software approximates and outputs total section area and total area of the infarct. The infarct size was expressed as a percentage of total ventricular area (%).

**Hemodynamic measurements:** Hemodynamic studies were performed 4 weeks after coronary ligation. The rats were anesthetized, and hemodynamic data were recorded by a recording system (Power Laboratory ML 845/4 channels, Biological Instruments, Besozzo, Italy). Briefly, a microtip pressure transducer catheter (Millar Instruments, Houston, TX, USA) was inserted into the right carotid artery and advanced into the LV to measure following parameters: LV end-diastolic pressure (LVEDP), LV maximum dP/dt (max dP/dt), LV minimum dP/dt (min dP/dt) and percentage of LV fractional shortening (%FS).

**Immunohistochemical staining:** To detect capillary endothelial cells in the infarct area, the harvested LV myocardium was embedded in OCT compound (Miles Scientific) and sectioned into 6-μm slices transversely. Tissue sections were stained with antibodies against rat CD31 (Abcam Biochemicals, Cambridge, UK). Capillary density was defined as CD31+ endothelial cells per high-power field (200×). Five fields were counted per section, with 10 sections per heart, and 10 hearts per group.

**Analysis of enzyme activities:** Four weeks after coronary ligation, the cardiac tissue in infarction area was dissected and preserved at -80°C for subsequent analysis. One-tenth of samples were used for the determinations of superoxide dismutase (SOD) activity, malondialdehyde (MDA) level in LV myocardium using the corresponding assay kit (both purchased from Sigma-Aldrich) according to the manufacturer’s instructions by highly sensitive ELISA spectrophotometry.

Briefly, for the determination of SOD activity, the IC50 (50% inhibition activity of SOD) values were determined under 450 nm. The results were expressed as units of SOD per milligram protein (U/mg protein).

For the determination of MDA levels, lipid peroxidation was detected by the reaction of MDA with thiobarbituric acid to form a colorimetric product (532 nm), which is proportional to the MDA present.[14] The results were expressed as nanomole MDA per milligram protein (nmol/mg protein).

**NO generation measurement:** The level of NO in MI cardiac tissue was detected by measuring the concentration of nitrite, a stable metabolite of NO, with the Griess reaction.[15] Briefly, fresh cardiac tissue in infarction area was homogenized in PBS with protease inhibitors at and centrifuged at 10,000 × g for 30 minutes at 4°C. Then the supernatant was collected and stored at -80°C before use. Before detection, duplicates of 100 μL of supernatant in each group was added to a 96-well microtiter plate and mixed with 100 μL of the modified Griess reagent (Sigma-Aldrich). After 15-minute incubation at room temperature, the plate was read on a spectrophotometer (Beckman, US-640 UV) at 540 nm. Quantification of nitrite was performed referencing to a standard curve constructed with increasing concentrations of sodium nitrite. The results were expressed as nanomole NO per milligram protein (nmol/mg protein).

**Western blot assay:** The myocardium was lysed in 0.2-mL RIPA buffer containing protease inhibitor cocktail (Sigma, St Louis, MO, USA) and 25 mM NaF as phosphatase inhibitor. Miscible liquids were centrifuged at 12,000 g for 15 minutes at 4°C. The quantity of total protein was assessed by a BCA protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China). Then 30 μg of protein was separated by 10% SDS-PAGE and transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA) before blocking with 5% skim milk for 2 hours at room temperature. Membranes were incubated with anti-inducible nitric oxide synthase (iNOS; 1:1,000, Santa Cruz, CA, USA), anti-endothelial nitric oxide synthase (eNOS; 1:500, Santa Cruz, USA) and anti-GAPDH (1:3,000, Santa Cruz, USA) overnight at 4°C. The primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies (1:2,000, Bio-Rad labora-
Crocin treatment significantly increased mean arterial blood pressure and LV end-diastolic pressure (LVEDP) (Supplemental Figure 2C). Moreover, crocin treatment significantly reduced infarction size (Supplemental Figure 2A) and improved hemodynamic parameters (Supplemental Figure 2B-E) in MI rats 8 weeks after MI.

**Crocin-induced angiogenesis in MI hearts**: To assess the angiogenesis effect of crocin, we measured capillary density in the infarction region of the left ventricle at 4 weeks after modeling. Immunohistochemistry staining was conducted with antibody against CD31 to detect endothelial cells in the capillaries of MI hearts (Figure 3A). As shown in Figure 3A and B, crocin significantly increased the capillary density in a dose-dependent manner, suggesting that crocin might promote the angiogenesis of infarct area. It should be noted that crocin treatment (60 mg/kg) did not alter capillary densities in the peri-infarction area (Supplemental Figure 3).

**Crocin exerted antioxidant effect in MI rats**: To further elucidate the underlying mechanisms of the protective effects of crocin, the factors associated with oxidative stress reported in MI, SOD, MDA, and NO were determined. Crocin significantly increased SOD activity (Figure 4A) while decreased MDA level (Figure 4B) both in a dose-dependent manner, indicating that crocin could exert antioxidative stress effect by activating the antioxidative enzyme SOD and decreasing MDA accumulation in MI hearts of rats. Crocin at low dose (20 mg/kg) did not influence NO production in the model rat, while crocin at 40 mg/kg or higher significantly decreased NO production (Figure 4C), suggesting that crocin could inhibit the generation of cytotoxic NO thus reducing the injury induced by excessive NO production.

**Crocin restored the balance between iNOS and eNOS of myocardium in model hearts**: NO production is mainly regulated by the balance between iNOS and eNOS. The expressions of iNOS, eNOS, and p-iNOS proteins, and phosphorylation of iNOS and eNOS in myocardium were determined by Western blot. As shown in Figure 5A and B, the expression levels of iNOS and p-iNOS were significantly decreased by crocin treatment compared with the control group in a dose-dependent manner. While eNOS and p-eNOS expressions were significantly increased in MI rats treated with low-to-high doses of crocin. Furthermore, the expression of F4/80 (macrophage marker) was unchanged by crocin treatment. These results suggested that crocin decreased iNOS and p-iNOS expressions in the LV myocardium to inhibit the harmful effect of iNOS on the myocardium, and increased eNOS and p-iNOS expressions in the myocardium.
Crocin treatment at indicated doses (20, 40, or 60 mg/kg) enhanced capillary densities in the infarction area. A: Representative images of CD31+ capillaries in the four groups. Scale bar, 50 μm. B: CD31+ capillary density was characterized by the number of capillaries per mm² area. Data were shown as mean ± SD. **P < 0.01 versus no crocin treatment group (Group 0 crocin).

eNOS expressions to exert its protective effects. Down-regulation of iNOS and upregulation of eNOS expressions would suppress the excessive production of NO and eventually attenuate the injury to the myocardium.

Discussion

In this study, for the first time, we investigated the protective effects of crocin on myocardial infarction in vivo using an experimental animal MI model and found that crocin exerted a protective effect by decreasing infarct size, improving hemodynamic parameters, inducing angiogenesis and enhancing the related antioxidant enzyme indexes. In consideration of above results, the antioxidant properties might be the mechanism underlying the antimyocardial infarction injury function of crocin.

Pharmacological studies have demonstrated the powerful antioxidant property for crocin, which is one of the main active constituent extracted from saffron.7,16,17) Crocin exerted protective effects against functional disturbances, oxidative stress, and tissue damages induced by gentamicin in rats.18) Crocin showed protective effect against haloperidol induced tardive dyskinesia due to its antioxidant activity.19) Crocin has been reported to exert antiapoptotic, antioxidant, and protective effects in cerebral12,20) retinal,17) and renal10 ischemia-reperfusion injuries.

To explore the protective effects of crocin on MI hearts, the MI rat model was established by the ligation of the left anterior descending coronary artery and verified by ST elevation in ECG in this study. The animals were intraperitoneally injected with crocin at indicated doses for seven successive days. 4 weeks after coronary ligation,
infarct size, hemodynamic parameters, and capillary density were determined. Crocin significantly attenuated infarct size (Figure 1), suggesting that crocin had an obvious cardioprotective effect and could promote myogenesis in the infarcted myocardium.

Hemodynamic studies showed that crocin improved hemodynamic parameters (Figure 2), indicating that crocin might improve the global cardiac dysfunction, consistent with a previous study that crocin had protective effects on hemodynamic parameters after ischemia reperfusion in isolated rat hearts. Capillary density was significantly increased in crocin-treated group (Figure 3), implying that crocin could promote angiogenesis in the MI myocardium, which might explain the improved hemodynamic parameters in crocin-treated MI rats. These results illustrate that crocin exerts anti-MI effects by enhancing myogenesis and angiogenesis in MI hearts.

Oxidative stress is regarded as one of the most important contributing factors in the development and pathogenesis of cardiovascular diseases. Evidences clearly indicate that heart failure is accompanied by excessive generation of ROS and depletion of endogenous antioxidant system. Low levels of antioxidant enzymes result in higher susceptibility to oxidative stress in the heart than in other organs. SOD, one of the enzymatic scavengers of ROS, is the first line of defense against the accumulation of ROS to limit the oxidative injury. MDA is an index to assess the extents of lipid peroxidation reactions. SOD activity and MDA level are correlated with the antioxidant responses. Either administration of exogenous antioxidants or upregulation of endogenous antioxidants could prevent MI-induced injury against ROS. This study indicates that crocin injection could enhance SOD activity as well as decrease MDA level in MI hearts (Figure 4A and B), implying that crocin exerts its cardioprotection on MI through an antioxidant pathway.

NO plays a critical role in the regulation of cardiac functions during ischemia, and excessive NO production may exert adverse effects on cardiovascular functions. NO is synthesized from its precursor L-arginine by a family of NOSs, including neuronal NOS, iNOS, and eNOS. The activation of NOS is concomitant with oxidative stress, resulting in NOS uncoupling to trigger further oxidative/nitrative stress. The activation of iNOS within vascular smooth muscle cells is the major factor causing hypotension in septic shock. In cardiac ischemia state after MI, expression of iNOS is upregulated, which generates a great deal of NO that either directly interacts with other factors or indirectly participates in scar formation and evolution process by adjusting fibroblasts, endothelial cells, and other functions. iNOS and eNOS are thought to participate in several cardiovascular disease processes.

Figure 4. Effects of crocin on SOD activity (A), MDA level (B), and NO level (C) in the myocardium in the infarction area of MI rats. Data were shown as mean ± SD. *P < 0.05 and **P < 0.01 versus no crocin treatment group (Group 0 crocin).

Figure 5. Effects of crocin treatment on expressions of iNOS, eNOS, phosphorylation of iNOS and eNOS, and F4/80 in the myocardium in the infarction area in MI rats. A: Protein expressions were analyzed by Western blot. GAPDH was used as an internal control. B: Relative protein expressions of these observed proteins were normalized to those of no crocin treatment group. Data were shown as mean ± SD. *P < 0.05 and **P < 0.01 compared with the no crocin treatment group (Group 0 crocin).
such as hypertension, atherosclerosis, heart failure, and MI injury. A body of evidences suggests that eNOS exerts a protective effect, whereas, in contrast, iNOS exhibits a harmful effect on the myocardium. Selective pharmacological inhibition of iNOS in MI/R injury is expected to be of significant therapeutic benefit, since it would maintain the physiological functions of eNOS while at the same time inhibit the generation of cytotoxic NO. Our results show that treatment with crocin attenuates NO production in the LV myocardium and blocks iNOS upregulation and eNOS downregulation, indicating that crocin restores the balance between iNOS and eNOS in the myocardium of MI hearts.

Interpretation of our results, however, should take into account some limitations. The main limitation is that the study is performed in an experimental rat model, allowing us to make speculations that cannot be directly extrapolated to human patients. Also the effects of crocin on normal rats were not determined. Moreover, we used one injury model and low-to-moderate doses of crocin.

Conclusions

This study demonstrates that intraperitoneal injection of crocin improves cardiac function after myocardial infarction, which enhanced myogenesis and angiogenesis in the infarcted myocardium. The antioxidant effect and restoration of the balance between iNOS and eNOS might contribute to the possible underlying mechanisms by which crocin takes part in the above process.

Disclosures

Conflicts of interest: The authors declare no conflicts of interest.

References


Supplemental Files
Supplemental Table
Supplemental Figures 1, 2, and 3
Please see supplemental files; https://doi.org/10.1536/ihj.17-114