Protective Effect of Crocin against Cerebral Ischemia in a Dose-dependent Manner in a Rat Model of Ischemic Stroke

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Background: Crocin is a water-soluble carotenoid isolated from the Crocus sativus L (saffron) stigma. It has previously been reported that it has protective effects against renal, cardiac, and global cerebral ischemic injury. However, its therapeutic effects remain to be clarified regarding ischemic reperfusion injuries, brain edema, and activity of antioxidant enzymes in a transient model of focal cerebral ischemia. Methods: Transient focal cerebral ischemia was induced by 60-minute middle cerebral artery occlusion (MCAO), followed by 23-hour reperfusion. Crocin at doses of 15, 30, 60, and 120 mg/kg intraperitoneally were injected at the start of ischemia. Infarct volume and neurologic outcome were evaluated 24 hours after MCAO. For the therapeutic time window measurement, crocin (60 mg/kg) was given 1, 3, and 6 hours after ischemia; 24 hours later brain edema and antioxidant enzyme activity were assessed. Results: The results indicated that treatment with crocin at doses of 30, 60, and 120 mg/kg significantly decreased infarct volume by 64%, 74%, and 73%, respectively. Administration of crocin (60 mg/kg) 1 hour before, at the start, or 1 hour after ischemia reduced brain edema by 48%, 52%, and 51%, respectively. Moreover, crocin (60 mg/kg) significantly reduced malondialdehyde (MDA) content and increased activity of superoxide dismutase (SOD) and glutathione peroxidase (GPx) in the ischemic cortex (P<.001). Conclusions: Our findings indicate that crocin has protective effects against ischemic reperfusion injury and cerebral edema in a rat model of stroke. These effects of crocin may have been exerted primarily by suppression of the production of free radicals and increased antioxidant enzyme activity. Key Words: Crocin—brain edema—ischemic reperfusion injuries—antioxidant enzyme activity—focal cerebral ischemia—rat.

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this study was to determine the effects of various doses of crocin on brain injuries, cerebral edema, and activity of antioxidant enzymes (glutathione peroxidase [GPx] and SOD) as well as malondialdehyde (MDA) content in a transient focal cerebral ischemia model in rats.

Material and Methods

Animals

Adult male Wistar rats (320 ± 20 g) were obtained from the breeding colony of Semnan University of Medical Sciences, Semnan, Iran. All rats were housed in cages in a 12-hour light/dark cycle at 22 to 24°C, with food and water ad libitum. All protocols of the study were performed in accordance with the National Institutes of Health guidelines for Care and Use of Laboratory Animals.

Focal Cerebral Ischemia

Using chloral hydrate (400 mg/kg, Merck Group, Darmstadt, Germany) anesthesia, we induced focal cerebral ischemia in rats, as previously described. Using laser Doppler flowmetry (LDF) guidance (DRT4 laser Doppler perfusion and temperature monitor; Moor Instruments Devon, UK), a 3-0 nylon suture was introduced into the internal carotid artery and gently advanced until LDF showed a sharp decrease in the ipsilateral cerebral blood flow to less than 20% of baseline, indicating adequate occlusion of the middle cerebral artery (MCA). After 60 minutes of middle cerebral artery occlusion (MCAO), reperfusion was started by withdrawing the nylon thread for 23 hours. To monitor the regional cerebral blood flow, an LDF probe was positioned in direct contact with the right temporal bone after a limited dissection of the temporal muscle at the middle distance between the eye and the ear. To fix and prevent the displacement of the LDF probe, a bur hole (2-mm diameter) was drilled 5 mm lateral and 1 mm posterior to the bregma without injury to the dura mater. Local right cortical cerebral blood flow was continuously monitored 15 minutes before, during the occlusion, and up to 15 minutes after the reperfusion.

Infarct Volume

Twenty hours after ischemia, rats were decapitated and the brains rapidly removed and cooled in saline at 4°C for 10 minutes. They were then sectioned coronally into 7 2-mm-thick slices using a Brain Matrix (Zivic Instruments, Pittsburgh, PA). The slices were immersed in 2% 2,3,5-triphenyltetrazolium chloride solution (Sigma Aldrich, Munich, Germany), and kept at 37°C in a water bath for 15 minutes. These slices were then photographed separately using a digital camera (Cannon, Melville, NY) connected to a computer. Unstained areas were defined as infarct and measured using image analysis software (NIH Image Analyzer). The infarct volume of each slice was calculated by multiplying the infarcted area of the slice by its thickness. The total infarct volume of each brain was calculated as the sum of the infarct volumes of the 7 brain slices. The contribution of the edema to the infarct volume was corrected using the following formula, as previously described: corrected infarct volume = left hemisphere size − (right hemisphere size − measured infarct size).

Cerebral Edema

Cerebral edema was evaluated by determining brain water content (BWC). Twenty-four hours after MCAO, the rats were killed and the brains were removed. Afterward, the pons and olfactory bulb were removed and the brains were weighed to obtain their wet weight. Subsequently, brains were dried at 110°C for 24 hours to determine their dry weight. BWC was calculated using the following formula: (wet weight − dry weight)/wet weight × 100.

Preparation of Tissue Homogenates

Brain was removed and ischemic cortex carefully isolated; subsequently, cortex was washed in cold 9% control and kept at −70°C. A fraction of cortex tissue was homogenized (1:10 w/v) in cold 1.15% KCl. The supernatants obtained after centrifugation at 20,000 × g for 10 minutes at 4°C were used for biochemical analyses. The level of total protein in supernatants was determined by the Bradford method using bovine serum albumin as standard.

Measurement of Malondialdehyde

The MDA content or thiobarbituric acid–reactive substances in ischemic brain cortex homogenates were measured using the thiobarbituric acid method as described previously. The initial sample of 250 mL of supernatant with 1.5 mL of 1% phosphoric acid and thiobarbituric acid 6% were mixed and incubated in a water bath for 45 minutes. After cooling, 2 mL of n-butanol was added to the mixture and mixed in a vortex mixer for 1 minute followed by centrifugation at 3000 rpm for 10 minutes. The organic layer was transferred to a fresh tube and its absorbance was measured at 532 nm using a spectrophotometer (Spectronic 1201, Milton Roy, Ivyland, PA). The standard calibration was plotted using 1,1,3,3-tetramethoxypropane. MDA concentration was expressed in nanomoles/milligram protein.

Measurement of Superoxide Dismutase and GPx Activity

Superoxide dismutase (SOD) and GPx activity in ischemic brain cortex homogenates were measured using a commercial kit (Randox Laboratories Ltd, Crumlin, UK) according to the manufacturer’s instructions.
The method uses xanthine and xanthine oxidase to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye. The SOD activity was assayed spectrophotometrically (Stat Fax 3300; Awareness Technologies, Palm City, FL) at 505 nm. The inhibition of the chromogen produced is proportional to the activity of the SOD present in the sample. A 50% inhibition is defined as 1 unit of SOD.

GPx activity was measured in homogenates using the method described by Paglia and Valentine. GPx catalyzed the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase (GR) and nicotinamide adenine dinucleotide phosphate, the oxidized glutathione was immediately converted to its reduced form with a concomitant oxidation of the reduced form of nicotinamide adenine dinucleotide phosphate to nicotinamide adenine dinucleotide phosphate. The decrease in absorbance was measured at 340 nm.

Measurement of Tissue Total Antioxidant Capacity

The ferric-reducing antioxidant power (FRAP) assay is a colorimetric method for measuring the total antioxidant capacity. Briefly, 1.5 mL of working FRAP reagent (25 mL .3 M sodium acetate buffer, pH 3.6; 2.5 mL .01 M 2,4,6-tri(2-pyridyl)-1,3,5-triazine in .04 M HCl; 2.5 mL .02 M FeCl3 • 6H2O; preheated to 37°C) was mixed with 50 μL of supernatant. The mixture was incubated at 37°C for 5 minutes, and the absorbance was determined at 593 nm. FeSO4 solutions were used for calibration. FRAP values are expressed as μmol/mg of protein.

Neurobehavioral Test

Neurologic (sensorimotor) examination was performed 24 hours after ischemia as described previously. The sum of partial scores yielded the total neurologic score, with a maximum of 42 points and a minimum of 0 points in normal rats. The rats were assessed neurologically by an observer who was blinded to the animal groups.

Experimental Groups

Infarct volume measurements and neurobehavioral tests were performed 24 hours after MCAO in 5 different groups: (1) control (saline as vehicle, n = 9), (2) crocin at 15 mg/kg (n = 7), (3) crocin at 30 mg/kg intraperitoneally (n = 7), (4) crocin at 60 mg/kg (n = 7), and (5) crocin at 120 mg/kg (n = 7). Crocin was administered at the beginning of MCAO in these groups.

To evaluate the therapeutic time window of the most effective dose of crocin (60 mg/kg) on brain edema, we used 7 different groups: (1) sham operated (n = 7), (2) control (n = 9), and in groups 3 (n = 7), 4 (n = 7), 5 (n = 7), 6 (n = 7), and 7 (n = 7), crocin was administered 1 hour before, at the beginning, or after 1 hour, 3 hours, and 6 hours of MCAO, respectively.

MDA content and SOD and GPx activity in cortex ischemic tissue were assessed 24 hours after MCAO in 3 separate groups (n = 6 each): 1 sham operated (surgical procedure without MCAO) and 2 control and crocin (60 mg/kg). In these groups, crocin was administered at the beginning of MCAO and enzymes assay and MDA content measurements were performed 24 hours after ischemia.

Animals were randomly assigned to the different treatment groups and the investigator who performed surgery on the animal was unaware of the animal’s allocated group.

Statistical Analysis

Data are presented as mean ± standard error of the mean. Data (infarct volume, neurologic outcome, and brain edema) were analyzed by nonparametric Kruskal-Wallis analysis of variance on ranks followed by the Dunn test. Data from enzyme assays were analyzed by parametric 1-way analysis of variance, followed by the Holm-Sidak method as post hoc analysis. Differences were considered significant at P less than .05 (SigmaStat 2.0; Jandel Scientific, Erkrath, Germany).

Results

Regional Cerebral Blood Flow

In all groups, regional cerebral blood flow decreased to less than 20% of the baseline value during 60 minutes of MCAO (Fig 1). There are significant differences between regional cerebral blood flow during MCAO in the groups treated with crocin at doses of 60 and 120 mg/kg compared with the control group (Fig 1, P < .05). The temporary occlusion by filament and under LDF monitoring in our study is likely to cause complete vessel occlusion and more reproducible lesion volume.

Figure 1. Regional cerebral blood flow (rCBF; % from baseline) before and during middle cerebral artery occlusion (MCAO) and after reperfusion in control (saline) and crocin-treated groups at doses of 15 (Crocin-15), 30 (Crocin-30), 60 (Crocin-60), and 120 (Crocin-120) mg/kg intraperitoneally. Abbreviation: SEM, standard error of the mean.
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Dose-Dependent Effect of Crocin on Infarct Volume and Neurologic Outcome

The infarct volume in the control group was 201 ± 8 mm³. Administration of crocin at the beginning of ischemia and at doses of 30 mg/kg (71 ± 3 mm³), 60 mg/kg (51 ± 7 mm³), and 120 mg/kg (52 ± 8 mm³) significantly decreased infarct volume by 64%, 74%, and 74%, respectively (P < .001) (Figs 2 and 3), whereas crocin at a dose of 15 mg/kg (128 ± 4 mm³) did not have a significant effect in reducing brain lesions compared with the control (saline as vehicle) group. Treatment with crocin at all doses reduced infarct area considerably in sections 2 to 5 in the posterior part of the MCA territory where cortical (ie, penumbral) tissue predominates (P < .001) (Figs 3 and 4).

The neurologic (sensorimotor) deficit score was 26 ± 1 at 24 hours after ischemia in the control group. Administration of crocin at the onset of ischemia and at doses of 30 mg/kg (17 ± 2), 60 mg/kg (11 ± 1), and 120 mg/kg (12 ± 2) improved neurologic outcome significantly (P < .001) (Fig 5), whereas crocin at a dose of 15 mg/kg (19 ± 2) did not change the neurologic score significantly compared with the control group (P > .05) (Fig 5).

Time Course Effect of Crocin on Cerebral Edema

The BWC in the left and right hemispheres of the sham operated rats were 78.2% ± .2% and 78.3% ± .16%, respectively. Induction of focal cerebral ischemia in the control group increased BWC of the ischemic hemisphere (right) significantly to 82.45% ± .29% (P < .001) (Fig 6).

Administration of crocin at the most effective dose (60 mg/kg) 1 hour before (80.7% ± .22%), at the start (80.7% ± .07%), and 1 hour after MCAO (80.95% ± .11%) reduced the postischemic increase of the BWC significantly compared with the control group (P < .001) (Fig 5). When crocin was administered 3 or 6 hours after ischemia, it had no significant effect in reducing the percentage of BWC (P > .05) (Fig 6). Furthermore, no significant difference in the percentage of BWC was found in the nonischemic hemisphere between the groups (Fig 6).

Effect of Crocin on the Activity of SOD, GPx Enzymes, Total Antioxidant Capacity, and MDA Content

Twenty-four hours after surgery, MDA content in brain cortex tissue was 2.28 ± .17 nmol/mg protein in the sham operated group. Ischemia increased MDA content significantly in the ischemic cortex of the control group (3.6 ± .1 nmol/mg protein; P ≤ .001). Treatment with crocin (60 mg/kg) at the start of ischemia caused a significant reduction in MDA content (2.95 ± .14 nmol/mg protein; P ≤ .001) (Fig 7A).

SOD enzyme activity in the cortex of the brain tissue was 21 ± .35 U/mg protein 24 hours after surgery in the sham operated group. Ischemia decreased SOD enzyme activity significantly (15 ± .4 U/mg protein) in the cortex tissue. Treatment with crocin (60 mg/kg) at the start of ischemia increased SOD enzyme activity significantly (16 ± .57 U/mg protein) in the ischemic cortex tissue (P ≤ .001) (Fig 7B).

GPx enzyme activity in the cortex of the brain tissue was 31 ± .009 U/mg protein 24 hours after surgery in the sham operated group. Ischemia decreased GPx enzyme activity significantly (26 ± .008 U/mg protein) in the cortex tissue. Treatment with crocin (60 mg/kg) at the start of ischemia increased SOD enzyme activity significantly (30 ± .007 U/mg protein) in the ischemic cortex tissue (P ≤ .001) (Fig 7C).

Total antioxidant capacity (TAC) in the cortex of the brain tissue was 1.3 ± .7 μmol/mg protein 24 hours after surgery in the sham operated group. Ischemia decreased FRAP significantly (.9 ± .03 μmol/mg proteins) in the cortex tissue. Treatments with crocin (60 mg/kg) at the start of ischemia increased TAC significantly (1.3 ± .06 μmol/mg protein) in the ischemic cortex tissue (P ≤ .001) (Fig 7D).

Discussion

Our results indicate that administration of crocin at the beginning of MCAO and at doses of 30, 60, and 120 mg/kg effectively reduced brain damage by 36%, 64%, 74%, and 74%, respectively. This finding suggests that the most effective dose of crocin is 60 mg/kg and implies that higher doses do not have more protective effect. Also, our results showed that crocin at doses of 60 and 120 mg/kg were equally effective, indicating a plateau in the response. This is in agreement with findings of another study that reported administration of crocin (10 mg/kg intravenously) at the onset of and 3 hours after focal ischemia reduced brain damage by 44% in mice. In that study, a single dose of crocin was administered to
mice, whereas in the present study, we evaluated the effect of different doses of crocin on brain damage, which is the major advantage of our work compared with that of Ochiai et al.3 Consistent with our findings, other researchers reported that crocin has a protective effect against brain damage in a global model of cerebral ischemia in rats.4,13

Another finding of ours is that local cerebral blood flow diminished to less than 20% of the baseline during 60 minutes of MCAO in all the experimental groups. This finding shows that with laser Doppler–guided filament placement, focal ischemia was induced with high confidence in all rats. However, without continuous LDF, it is difficult to judge whether the MCA was adequately occluded.22 Moreover, our results showed that local cerebral blood flow in the groups treated with crocin (60 and 120 mg/kg) decreased significantly compared with the control group. Our pilot study revealed that crocin has a hypotensive effect at higher doses and may lead to severe lowering of blood pressure, as supported by other studies.23 Because autoregulation mechanisms of cerebral blood flow are impaired after stroke,24 hypotension induced by crocin may lead to a decrease of cerebral blood flow. It is an interesting observation that despite lowering cerebral blood flow, crocin retains protective effects against brain damage.

We also demonstrated for the first time, to our knowledge, that at its most effective dose (60 mg/kg), crocin suppressed posts ischemic brain edema considerably with a 1-hour therapeutic window. This is an interesting finding, indicating that the antiedematous effects of crocin persist for 1 hour after ischemia. Although there is no similar study, some indirect evidence suggests protective effects of crocin against blood-brain barrier damage after global cerebral ischemia in mice,4 which could somewhat confirm the results of our study. Our previous study showed that the compromising of blood-brain barrier permeability during ischemia may lead to reduction of brain edema in a transient model of focal cerebral ischemia.14

The most important issue in patients who have experienced stroke is neurobehavioral outcome rather than infarct volume. Thus, along with infarct size analyses,
measuring the neurobehavioral state is essential for preclinical assessment of the potential therapeutic procedures for stroke.

The findings of this study revealed that at all doses (except 15 mg/kg), crocin improved neurologic outcome significantly, along with a decrease in cerebral infarct volume. Since crocin is a pharmacologically active component of *Crocus sativus L* (saffron), this finding is to some extent similar to the report of another study stating that oral administration of saffron a week before focal cerebral ischemia significantly improved neurobehavioral outcome in rats.12

Under pathologic conditions such as ischemia, the balance between oxidant and antioxidant systems is jeopardized.4,12 Oxidative stress and the production of free radicals constitute a main cause of interruption of the blood-brain barrier and formation of cerebral edema after cerebral ischemia.26 The results of our study indicate that crocin prevents the increase of MDA levels, a marker of lipid peroxidation, and also increases SOD and GPx enzyme activity considerably after cerebral ischemia in the cortex. This finding suggests antioxidant properties for crocin as well as the capacity to restore SOD and GPx activity effectively after stroke. Consistent with our results, a previous study demonstrated that at the same concentration, crocin is a more effective antioxidant than z-tocopherol.27 Therefore increasing the capacity of cellular antioxidant enzymes and decreasing the production of free radicals by crocin may protect cells against oxidative stress damage after cerebral stroke, as found in this study.

Some evidence suggests that tumor necrosis factor (TNF)-α is activated during brain ischemia.28,29 It causes damage to cerebral endothelial cells30 and increases blood-brain barrier permeability,31 thus contributing to brain edema formation. Therefore we propose that the antiedematous effects of crocin are partly due to an inhibition of TNF-α synthesis and, consequently, the protection of the blood-brain barrier against disruption.4 Moreover, recently researchers have reported that proinflammatory cytokines such as interleukin (IL)-1β and IL-6 and activation of NF-κB play important roles in the pathogenesis of brain edema.32,33 Recently, Nam et al6 reported that crocin effectively reduced lipopolysaccharide-stimulated production of IL-1β, TNF-α, and NF-κB activation in cultures of hippocampal slices.6 This suggests that the beneficial effect of crocin may result, at least in part, from inhibiting the production of these cytokines and, consequently, their signaling pathways. Further research is needed to elucidate these possibilities. In addition, some evidence indicates that free radicals and oxidants play pivotal roles in the development of edema and blood-brain barrier disruption after cerebral ischemia.26 According to our results and previous studies,4,27 it seems that part of the beneficial effects of crocin in reducing edema and brain lesions may be related to its effect in curbing the production of free radicals and antioxidant properties. We cannot exclude the possibility that part of the antiedematous effects of crocin might be related to its effect in reducing brain lesions.

As reported previously, crocin has very low toxicity in rats, even at high dosages.6,34 Nonetheless, the safety of crocin should be evaluated precisely for clinical applications. The results of the present study are in favor of considering crocin as a new pharmacologic tool for treating stroke in humans.

In conclusion, the present study indicated that crocin has protective effects against ischemic reperfusion injury and cerebral edema in a rat model of transient focal cerebral ischemia. These effects of crocin may have been exerted mainly by suppressing the production of free radicals.
radicals as well as increasing the capacity of cellular antioxidant enzymes.

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References