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Neuroprotective effects of crocin on the histopathological alterations following brain ischemia-reperfusion injury in rat

Javad Raouf Sarshoori [1], Mohammad Hossien Asadi [1], Mohammad Taghi Mohammadi [2]*

[1] Department of Anatomy, Faculty of Medicine, Baqiyatallah University of Medical Sciences, Tehran, Iran
[2] Department of Physiology and Biophysics, Faculty of Medicine, Baqiyatallah University of Medical Sciences, Tehran, Iran

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ABSTRACT

Objective(s): Some histopathological alterations take place in the ischemic regions following brain ischemia. Recent studies have demonstrated some neuroprotective roles of crocin in different models of experimental cerebral ischemia. Here, we investigated the probable neuroprotective effects of crocin on the brain infarction and histopathological changes after transient model of focal cerebral ischemia.

Materials and Methods: Experiment was performed in four groups of rats (each group; n=8), sham, control ischemia and ischemia treated rats. Transient focal cerebral ischemia was induced by 80 min middle cerebral artery occlusion (MCAO) followed by 24 hr reperfusion. Crocin, at doses 50 and 80 mg/kg, was injected at the beginning of ischemia (IP injection). Neurologic outcome (Neurological Deficit Score, NDS scale), infarct volume (TTC staining) and histological studies were assessed 24 hr after termination of MCAO.

Results: Treatment with crocin, at doses 50 and 80 mg/kg, significantly reduced the cortical infarct volume by 48% and 60%, and also decreased striatal infarct volume by 45% and75%, respectively. Crocin at two different doses significantly improved the NDS of ischemic rats. At histological evaluation, crocin, at dose 80 mg/kg more than 50 mg/kg, decreased the number of eosinophilic (prenecrotic) neurons and reduced the fiber demyelination and axonal damage in ischemic regions.

Conclusion: Our findings indicated that crocin effectively reduces brain ischemia-induced injury and improves neurological outcomes. Crocin also is a potent neuroprotective factor that can be able to prevent histopathological alterations following brain ischemia.

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Introduction

Stroke is the third cause of death and the first cause of disability in the world (1). Reduction or cessation of blood flow in ischemic area causes the uncontrolled neuronal depolarization, inflammatory responses, overproduction of free radicals, acidosis and increased intracellular calcium that lead to neuronal death (2). Based on histological studies, some histopathological alterations take place after brain ischemia. The study of Pantoni *et al*, has shown the appearance of necrotic neurons in the cortical and striatal area, vacuolation and pallor of the white matter and segmental swelling of myelinated axons after focal brain ischemia (3). The findings of another study have demonstrated the increment of oligodendrocyte precursor cells numbers at the margins of infarct area (4). Other investigations also have shown the neuronal necrosis and various glial responses after transient middle cerebral artery (MCA) occlusion (5). Based on study of Degrolami *et al*, neuronal necrosis, white matter and vascular lesions take place after permanent focal cerebral ischemia (6). Finally, different human studies have shown the major neurological complications and histopathological changes including neuronal, glial and vascular damages in acute ischemic stroke (7, 8).

Crocin is one of the most important constituents of saffron (9). It is a water soluble carotenoid with strong antioxidant activity (10-12). Many studies have shown that crocin has many potential therapeutic effects such as anti-apoptotic (13), anti-inflammation (14) and anti-hypertensive actions (15, 16). *In vitro* studies have shown that crocin has prevented from cell death due to oxidative stress (17). It also has neuroprotective effect on acrylamid-induced apoptosis in PC-12 cells (13) and light- induced photoreceptor death in primary retinal cell culture (18). The findings of a new study have demonstrated that crocin has diminished

*Corresponding author: Mohammad Taghi Mohammadi. Department of Physiology & Biophysics, School of Medicine Baqiyatallah University of Medical Sciences, Tehran, Iran. Tel/Fax: +98-21-26127257; email: Mohammadi.mohammadt@yahoo.com, Mohammadimohammadt@bmsu.ac.ir

neuroinflammation and neurodegeneration of autoimmune encephalomyelitis by preservation of myelin and axonal density in spinal cords (14). Different *in vivo* studies also have revealed that crocin is able to attenuate ischemia-reperfusion induced damages in kidney(19), muscles (20), retina (18) and brain (21). Also crocin improved learning and memory impairments in diabetic rat (12). On the other hand, ultrastructural assessment has shown crocin decreased reperfusion-induced oxidative/nitrative injury to cerebral microvessels after global cerebral ischemia in mice (22). Also histopathological study has revealed crocin reduced wallerian degeneration of sciatic nerve crush-injured in rat (10).

It is well known that histopathological alterations take place after ischemic stroke. Since there is no study to evaluate the effects of crocin on the brain tissue damage following ischemia-induced injury, we investigated the effects of crocin on the histopathological alterations and cerebral injuries after focal brain ischemia-reperfusion injury in rat.

Materials and Methods

Animals

Adult thirty two male Wistar rats, weighing 270-320 g, were obtained from the experimental animal center, Baqiyatallah University of Medical Sciences, Iran. Animals were kept with free access to food and water, at 23±2˚C, and a 12 hr light/dark cycle throughout the study. All experimental protocols were in accordance with Animal Care Committee Guidelines of Baqiyatallah University of Medical Sciences.

Experimental design

Rats were randomly divided into four groups (each group=8), sham, control ischemia (I/R), ischemia treated by crocin (Sigma Aldrich, Germany) with doses 50 and 80 mg/kg (I/R+crocin 50 and I/R+crocin 80). Rats received crocin via IP injection at the beginning of ischemia.

Focal cerebral ischemia-reperfusion model

Focal brain ischemia was induced by transient occlusion of middle cerebral artery (MCA), as previously described in detail according to Longa method (23). Rats were anesthetized with 2.5% isoflorane (Forane, UK) and placed in dorsal recumbence. The right common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were exposed via a midline pretracheal incision. The vagus and sympathetic nerves were carefully separated from the arteries. A 3-0 nylon suture (Ethicon, UK) with rounded head by heating over flame, was inserted from the lumen of the ECA to that the ICA until a mild resistance was felt. Thereby, the origin of right MCA was occluded. After 80 min MCA occlusion, reperfusion phase was started by withdrawing the nylon thread for 24 hr. During and after the surgery, a heating pad and heating lamp were used to maintain the rectal temperature between 36.5 to 37.5 and complete recovery of the animal from the anesthesia. In the sham group, animals were prepared in the same procedure except for the insertion of nylon thread into the right internal carotid artery.

Neurological evaluation

Neurological deficit score (NDS) were performed in both the control ischemic and ischemia treated rats after 24 hr of reperfusion by a single experimenter who was blinded to the experimental treatment groups. Neurological findings were scored on a 5-point scale, as previously described in detail by Plesnila (24). The criterion of the evolution was as follows: grade 1 no observable neurological deficit, grade 2 was given to rats that showed flexion of contra lateral torso or forelimb upon lifting by their tail, or failure to extend their forepaw when suspended vertically, forelimb flexion and shoulder adduction, grade 3 was for circling to the contralateral side of the MCA occluded hemisphere when the animal is held by the tail on a flat surface, but with normal posture at rest , grade 4 was assigned to loss of righting reflex and decreased resistance to lateral push, and finally, grade 5 was for no spontaneous motor activity.

Infarct volume

Twenty four hours after reperfusion, the rats decapitated and brain removed and cooled in normal saline at 4˚C for 10 min. They were then sectioned coronaly into 6, 2-mm-thick slices using a Brain Matrix. Slices were incubated in dark at 37˚C for 30 min in a solution of 2% 2, 3, 5-triphenyltetrazolium chloride (TTC, sigma) in a water bath. These slices were then photographed separately using a digital camera (canon, Japan). Unstained areas were defined as infarct and measured using image analysis software. The total infarct volume of each brain was calculated as the sum of the infarct volumes of 6 brain slices (25). The corrected infarct volume was calculated using the following formula:

Infarct volume= Left hemisphere size – (Right hemisphere size – Measured infarct size)

Tissue swelling

Twenty four hr after reperfusion the rats were killed and the brains were removed. The brain divided into two hemisphere then olfactory bulb and brain stem were removed and determined their total volume of hemispheres based on infarct volume method (25). Tissue swelling percentage was calculated using the following formula:

$$\%\text{Tissue swelling} = [(V_{\text{Right Hemisphere}} - V_{\text{Left Hemisphere}})/V_{\text{Left Hemisphere}}] \times 100$$

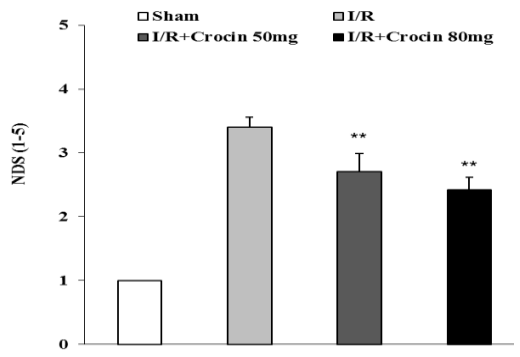

Figure 1. Neurological deficit score (NDS) of sham group after 24 hr surgery and ischemic groups (I/R and I/R treated groups) after 24 hr reperfusion. All values are mean±SEM.
Significantly different from Sham group (P<0.01)
** Significantly different from I/R group (P<0.01)

Histological assessment

Twenty four hr after surgery in sham group and twenty four hr after termination of ischemia in ischemic groups, animals were transcardially perfused with normal saline and followed by 4% PBS-buffered formaldehyde. The brains were removed and post-fixed in 4% PBS-buffered formaldehyde for 48 hr. After fixation and tissue processing, coronal serial sections (5 µm in thickness) prepared for conventional histologic examination. Paraffin embedded sectioning (each 50 µm intervals), processed routinely for hematoxylin and eosin (H&E) or Luxol fast blue (LFB) staining. The histological changes were observed through a light microscope (Nikon, Japan) and the presence of myelin damage was assessed by LFB staining (Figure 8). Eosinphilic neurons were defined as intensely acidophilic, triangular shape, major darkening and shrinkage of nucleus and cytoplasm (Figure 6). Four microscopic fields at 400X in the cortex and striatum of lesioned-hemispheres were captured and the numbers of eosinphilic neurons were counted. Quantification was performed by Motic software.

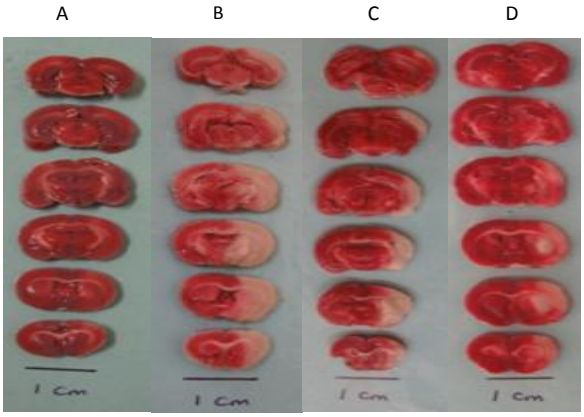

Figure 2. Photograph illustrating the coronal sections of rat brain stained with TTC in sham group (A) after 24 hr neck surgery, control ischemia (B) and ischemia treated groups [at doses 50 (C) and 80 (D) mg/kg] followed by 24 hr reperfusion. Non-ischemic areas are colored deep red whereas ischemic areas are white

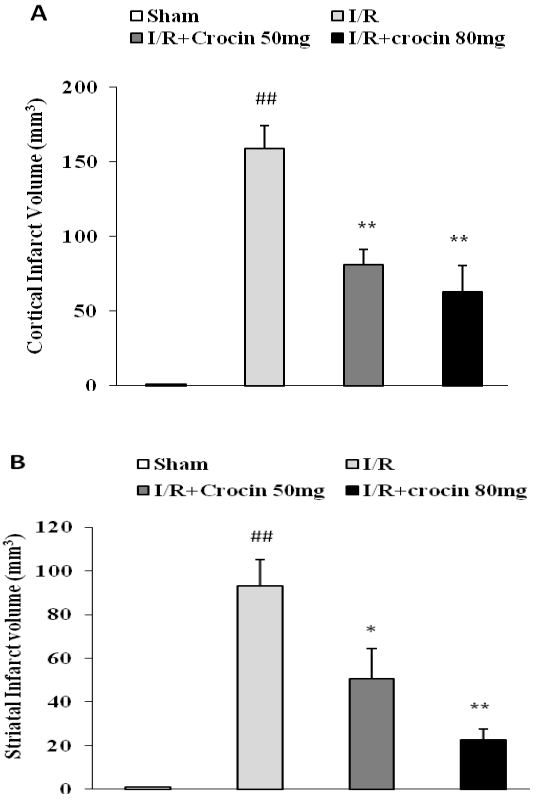

Figure 3. Infarct volume of sham group after 24 hr surgery and ischemic groups (I/R and I/R treated groups) after 24 hr reperfusion in the cortex (A) and striatum (B). All values are mean±SEM
Significantly different from Sham group (P<0.01)
* Significantly different from I/R group (P<0.05)
** Significantly different from I/R group (P<0.01)

Statistical analysis

All Data was presented as Means±SEM. One way ANOVA and Tukey *Post-hoc* test was used for analysis of data between different groups. Non-parametric test and Mann-Whitney U test also was used for NDS. P<0.05 was considered as significant differences.

Results

Neurological deficit score (NDS)

All animals were evaluated for their neurological deficits before test and 24 hr after surgery. There was no neurological deficits before beginning test and 24 hr after surgery in the sham group. MCAO induced neurological deficits after ischemia and 24 hr reperfusion in I/R group (3.40±0.16). Administration of crocin at doses 50 and 80 mg/kg significantly improved neurologic outcome compared with I/R group (P<0.01) (Figure 1).

Infarct volume

Qualitative assessment of infarct size of lesioned hemisphere (right) of all groups has shown at Figure 2. Photographs show that surgery in sham group did

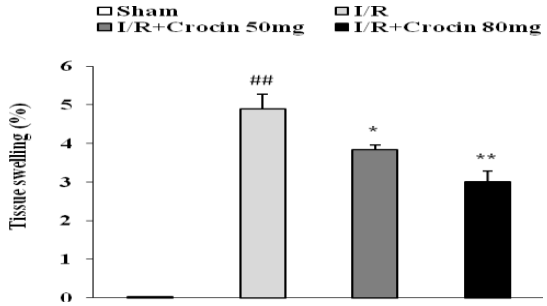

Figure 4. Tissue swelling percentage of sham group after 24 hr surgery and ischemic groups (I/R and I/R treated groups) after 24 hr reperfusion .All values are mean±SEM.
Significantly different from sham (P<0.01).
* Significantly different from I/R group (P<0.05)
** Significantly different from I/R group (P<0.01)

not induce any infarction in right hemisphere (A). But MCAO in control ischemia (I/R group) induced infarction in large area of cortex and striatum at right hemisphere (B). Comparison of white area in crocin treated rats at the beginning of ischemia indicated that crocin at doses 50 and 80 mg/kg reduced the infarct size in both cortex and striatum (Figure 2).

Quantitative measurement of infarction has shown in Figure 3. Infarction in sham group was zero but MCAO in control ischemia caused infarct formation. Our results revealed that the cortical and striatal infarct volume in I/R group was 159±15 mm^3 and 93±12 mm^3, respectively. Crocin administration at the beginning of ischemia at the dose of 50 mg/kg significantly decreased cortical (81±10 mm3) and striatal (50±14 mm3) infarct volume. Crocin also, at dose 80 mg/kg, significantly reduced cortical (62±18mm3) and striatal (22±5 mm3) infarct volume more than crocin at dose 50 mg/kg.

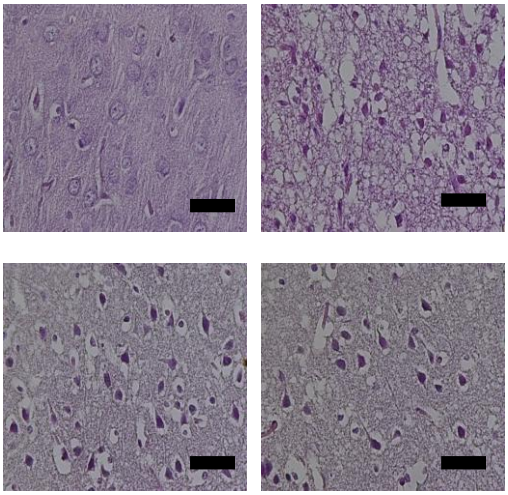

Figure 5. Photographs show the effects of crocin on histological changes (H&E staining) in the cortex of sham group after 24 hr surgery and ischemic groups (I/R and I/R treated groups) after 24 hr reperfusion. Crocin at the dose of 80 mg/kg more than the dose of 50 mg/kg reduced of pyknotic and shrinkage of nucleus with widened pericellular spaces, edema in neuropil following I/R injury-induced. A; Sham, B; I/R, C; crocin-treated rats at the dose of 50 mg/kg. D; crocin-treated rats at the dose of 80 mg/kg. (400X-scal bar 100 µm)

Tissue swelling

Tissue swelling percentage of brain hemispheres in sham group after 24 hr surgery was zero (Figure 4). Ischemia induced tissue swelling in ischemic groups after 24 hr reperfusion with different content. In control ischemia group, the value of tissue swelling was 4.9 %. Treatment with crocin at doses of 50 and 80 mg/kg significantly reduced the percentage of tissue swelling in treated rats by 3% and 3.8% compared with control ischemia, respectively (Figure 4).

Histological studies

Obtained photographs of the brain cortex, subcortical white matter (corpus callosum) and striatum (caudoputamen) were examined with light microscopy in ischemic hemisphere (right hemisphere). Histological assessment of brain coronal sections stained with H&E and LFB didn't show any histological alterations after 24 hr surgery in the sham group. Evaluation of ischemic hemispheres of control ischemia group revealed that 80 min MCAO and 24 hr reperfusion caused different lesions in brain tissue. The H&E stained micrographs showed the meningeal changes (subleptomeningeal edema, which is presented by enhanced space between arachnoid and pia matter, data not shown) neuropathological changes (heterogeneous neuronal changes, pyknotic and shrinkage of nucleus with widened pericellular spaces and eosinophilic neurons (characterized by condensed acidophilic cytoplasm, formation of triangular nuclear pyknosis), congestion, vacuolization and edema in neuropils (neuropils having vacuoles.) (Figure 5). Assessment of ischemic regions in treated groups showed that crocin attenuated the histopathological alterations of ischemic hemispheres more at the dose of 80 mg/kg compared to 50 mg/kg. In quantification assessment, crocin administration at doses of 50 and 80 mg/kg significantly decreased the number of eosinophilic neurons at ischemic area of ischemic treated rats (P<0.05, P<0.01) (Figure 6).

The brain tissue micrographs of control ischemic rats stained with LFB showed nerve fiber changes (demyelination and axonal fragmentation) in the subcortical white matter (Figure 7), and demyelination of nerve fiber bundles of the striatum (Figure 8). Assessment of ischemic regions in treated groups showed that crocin at the dose of 80 mg/kg more than 50 mg/kg attenuated demyelination and axonal fragmentation of ischemic hemispheres.

Discussion

The findings of the present study indicated that crocin administration, at the onset of ischemia, has reduced brain ischemia-induced injury. Our results also show that crocin has improved the neurological deficit of ischemic rats and decreased the tissue swelling percentage of the ischemic hemisphere.

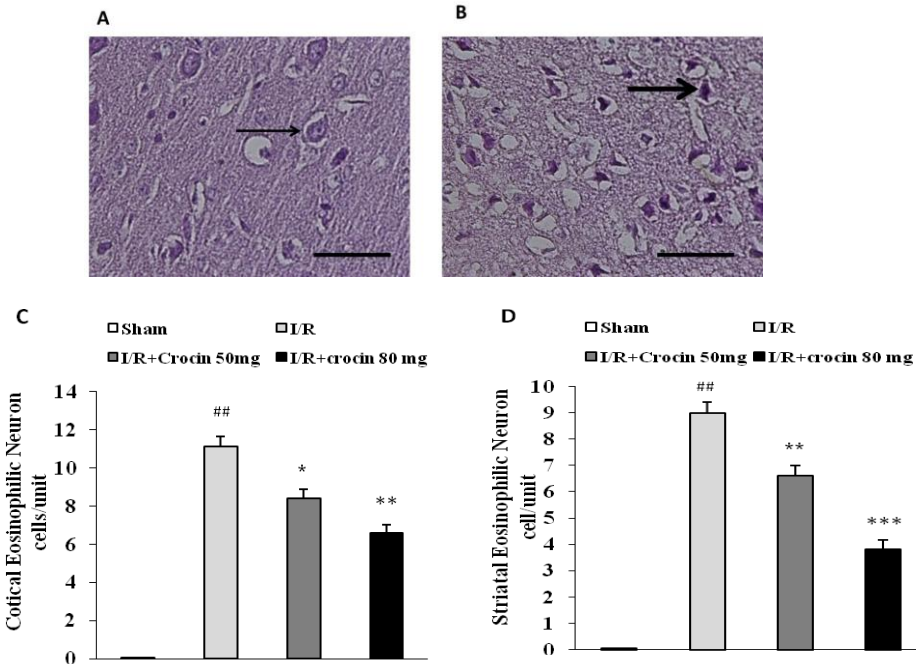

Figure 6. Number of eosinophilic neurons in the ischemic region of I/R groups. Photograph A and B show the H&E stained coronal sections of ischemic hemispheres in I/R groups (A: Normal neuron; thin arrow, B: Eosinophilic or red neuron; thick arrow, 400X-scale bar100µm). Graphs show the number of Eosinophilic neurons in the cortex (C) and Striatum (D) of all groups. All values are mean±SEM
Significantly different from sham (P<0.01); * Significantly different from I/R group (P<0.05); ** Significantly different from I/R group (P<0.01); *** Significantly different from I/R group (P<0.001)

Histological evaluations revealed the neuroprotective roles of crocin against the ischemia-induced neurodegeneration, and crocin administration before brain ischemia prevented histopathological changes and reduced the number of eosinophilic cells (Red neuron) in the ischemic region. Crocin also avoided from demyelination of fibers and interruption of nerve fiber bundles. These results pointed to the protective actions of crocin against brain ischemia.

Crocin is a water soluble constituent of saffron which has many medicinal properties such as anti-inflammatory, anti-edematous, nerve regeneration enhancing, microtubule polymerization and antioxidant effects (26, 27). Recent findings have indicated that crocin has a more power antioxidant property (11), and based on previous studies, antioxidants are one of the main interventions that are able to reduce the side effects of brain ischemia and ischemic stroke outcomes. There is normally a balance between production of free radicals and antioxidant defense system and impairment of this balance causes tissue oxidative stress (28). In the early stages of brain ischemia, the production of free radicals increases and plays an essential role to progress ischemia-induced brain damage. Also, enhancement of these radicals has an important role during reperfusion injury after transient ischemia (29, 30). The results of our study showed that treatment with crocin during brain ischemia was able to reduce the cortical and striatal infarct volume (Figure 3). According to the results of this study and some other similar investigations, it seems that high antioxidant property of crocin has leaded to reduction of infarct volume at ischemic areas of ischemic treated rats (21). The results of our study also show that crocin treatment, at the beginning of ischemia, improved neurological deficit. These findings reveal the protective actions of crocin against ischemia-induce neurological deficit, and there are better outcomes for ischemic rats that treated with crocin perhaps due to antioxidant property of crocin and reduction of neuronal death at ischemic regions. The results of our study also are indicating that crocin at the dose of 80 mg/kg has decreased infarction and improved neurological outcome more than the dose of 50 mg/kg. These findings suggest the more protective effects of higher doses. Our results differ with findings of vakili *et al*, which reported the most effective dose of crocin was 60 mg/kg and applying of higher doses did now show more protective effect (21). However, Ochiai *et al*, reported that intravenous injection of crocin (15 mg/kg) 3 hr after the onset of focal ischemia has decreased brain injury (31). Also, Zheng *et al*, showed the pretreatment of crocin for 21 days (5, 10, 20 mg/kg) has decreased the brain damage in global ischemia in mice (22).

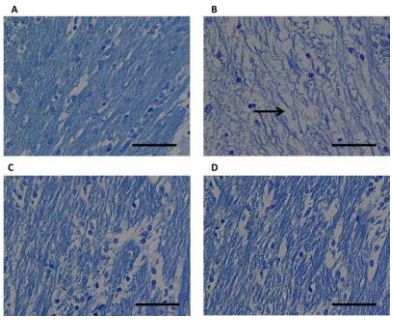

Figure 7. Photographs show the effects of crocin on histological changes (Luxol fast blue staining) in the subcortical white matter (corpus callosum) of sham group after 24 hr surgery and ischemic groups (I/R and I/R treated rats) after 24 hr reperfusion. Crocin at the dose of 80 mg/kg more than the dose of 50 mg/kg reduced rarefaction caused by demyelination following I/R injury-induced (arrow). A;Sham, B; I/R, C; crocin-treated rats at dose of 50 mg/kg. D; crocin-treted rats at the dose of 80 mg/kg. (400X-scal bar 100 µm)

It is well known that blood-brain barrier (BBB) interruption and edema formation following brain ischemia cause developing ischemia-induced injuries. Following edema formation, the intracranial pressure increases and compress the cerebral vessels and finally decreases the cerebral perfusion of ischemic regions (32). Therefore, prevention of edema formation after brain ischemia can be helpful for better ischemic stroke outcomes. In the present study, we showed significant tissue swelling after ischemia. However, the content of tissue swelling significantly was lower in crocin treated rats (Figure 4). Since the crocin acts as a powerful antioxidant, perhaps this property of crocin has reduced the tissue swelling of ischemia treated rats. Based on previous researches, variety of free radical production (oxygen and nitrogen free radicals) play an important role for BBB damage and edema after brain ischemia (33).The simultaneous production of these free radicals cause to production of peroxynitrite compound which is very toxic for BBB cells. These free radicals also contribute to brain matrix metalloproteinases (MMPs) activation (34), and activation of MMPs cause BBB disruption following brain ischemia.

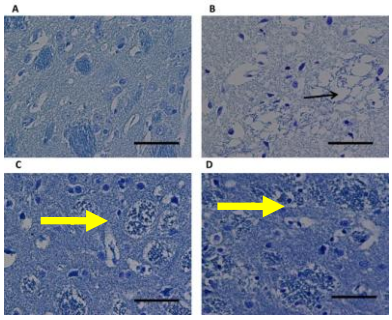

Figure 8. Photographs show the effects of crocin on histological changes (Luxol fast blue staining) in the striatum (caudoputamen) of sham group after 24 hr surgery and ischemic groups (I/R and I/R treated groups) after 24 hr reperfusion. Crocin at the dose of 80 mg/kg more than 50 mg/kg reduced demyelination of fiber bundles following I/R injury-induced (arrow). A; Sham, B; I/R, C; crocin-treated rats at dose of 50 mg/kg. D; crocin-treted rats at the dose of 80 mg/kg. (400X-scal bar100 µm)

The histological findings of present study showed considerable histopathological changes after 24 hr MCA occlusion. Pyknotic and eosinophilic neurons, enhancement of pericellular space and demyelination of axon fibers were the main alterations that were observed (Figures 5, 7, 8). These findings are accompanied with some other recent studies that have shown these histopathological alterations. The brain autopsied samples of stroke patients and animal models of ischemic stroke have reported the increment of perivascular space, eosinophilic neurons, ghost neurons and demyelination of axon fibers (8, 35). The study of Otilia *et al*, also showed that the eosinophil neurons and ghost cells can be observed at day 16 in monkey and even at day 35 to 60 in human following focal brain ischemia (7). Eosinophilic neurons are the classic appearance of neurons degeneration (36). The brain tissue contains many unsaturated fatty acids that can be easily peroxidated. The brain tissue also is susceptible to oxidative damage because it consumes a lot of oxygen and the activity of brain's antioxidant enzymes is weak (37). Therefore, because of free radical production following brain ischemia, the different cells of brain tissue are vulnerable to oxidative damage and histopathological changes. Also oxidative stress may contribute to axonal damage via the impairment of mitochondrial function (38). In the present study crocin administration before ischemia significantly decreased the histopathological changes. Also the number of eosinophilic neurons, size of pericellular space and demyelination of axon fibers were reduced in crocin treated rats (Figurss 6-8). These findings are in agreement with other studies that crocin prevented from neurodegeneration in different pathological conditions. Crocin, in crush-induced injury of sciatic nerve, improved motor behavior and prevented the histological changes (Wallerian degeneration) of distal segment of sciatic nerve (10). Also it prevented neurodegeneration of hippocampal neurons in diabetic rats (12). Another study has been established to evaluate the possible neuroprotective effects of crocin on traumatic brain injury in mice and they found out crocin was able to reduce brain edema and attenuated motor functional deficits (39). *In vitro* studies also demonstrated that crocin can reduce the production of various neurotoxic molecules from activated microglia (40). Based on mentioned studies, our results reveal the protective actions of crocin against ischemia-induced histopathological alterations, and there are better outcomes for ischemic rats that treated with crocin perhaps due to antioxidant property of crocin and finally, reduction of neuronal death at ischemic regions.

Conclusion

The findings of our study suggest that crocin effectively reduces ischemia-induced damage and improves neurological outcome. Also crocin, as a power neuroprotective factor, prevents the histopathological alterations following brain ischemia and reduced the neuronal damage. Accordingly, crocin can be candidate for an effective agent in reducing ischemic stroke-induced brain tissue damage alone or in combination with other drugs.

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