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Crocin protects against rat brain ischemia-reperfusion injury *in vivo* and the studies on certain aspects of the mechanisms involved*

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Abstract: Crocin pretreatment markedly improved the neurological dysfunction and decreased the infarct volume in a dose-dependent manner in middle cerebral artery occlusion (MCAO) rats. Western blot analysis showed that crocin up-regulated B-cell lymphoma 2 (Bcl-2) expression, down-regulated Bcl-2 associated protein X (Bax) and cleaved caspase-3 expression of hippocampus in MCAO rats. Because oxidative/nitrative stress is a very important factor in ischemia-reperfusion (I/R) injury, thus, a sodium nitroprusside (SNP) -impaired PC12 cell model was set up to mimick nitric oxide (NO) excitotocixity in I/R brain. The results showed that crocin protected PC12 cells against SNP-induced cytotoxicity via attenuating the caspase activation and mitochondrial dysfunction *in vitro*. Crocin significantly attenuated apoptosis, lactate dehydrogenase (LDH) release, caspase-3 activation, mitochondria membrane potential corruption and the intracellular accumulation of ROS induced by SNP in PC12 cells. Moreover, SNP decreased the expression level of Bcl-2, induced the expression of cytochrome *c* and Bax in PC12 cells, which is similar to the case of hippocampus in MCAO rats; and crocin reversed all these effects. All these evidences support that crocin is an effective agent to protect I/R injury via reasonable mechanism.

Key words: crocin; cerebral ischemia; ischemic-reperfusion (I/R); oxidative/nitrative stess; apoptosis; sodium nitroprusside
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藏红素在体保护大鼠脑缺血/再灌注损伤和可能的作用机制

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摘　要：通过中脑冠状动脉闭塞（MCAO）造成缺血/再灌注（I/R）损伤大鼠模型。藏红素预处理可以剂量依赖性地改善 I/R 损伤所造成的神经性机能障碍和降低脑梗死体积。免疫印迹分析显示藏红素能够上调 MCAO 大鼠大脑海马中 Bcl-2 的表达，下调 Bax 和 Caspase-3 的表达。由于氧化/硝化应激在 I/R 损伤中相当重要，建立了硝普钠（SNP）－诱导损伤的 PC12 细胞模型来模拟在 I/R 大脑中大量释放一氧化氮（NO）造成的神经毒性。研究结果与藏红素保护 MCAO 大鼠造成海马损伤一致，藏红素显著下调 SNP－损伤 PC12 细胞所引起 LDH 和 ROS 水平的升高，并且剂量依赖性下调促凋亡蛋白 Bax，Caspase-3 和 cytochrome *c* 水平的升高，而上调抗凋亡蛋白 Bcl-2 蛋白的表达。所有证据表明，藏红素是一种可能通过调控凋亡蛋白活性及线粒体机能障碍机制有效保护 I/R 损伤的药物。

关键词：藏红素；大脑缺血；缺血再灌注（I/R）；氧化/硝化应激；凋亡；硝普钠

Ischemic stroke is a kind of serious disease leading to death and disability which is induced by a reduction or even blockade of blood flow to the cerebrovascular system[1]. In this pathological process, a series of events including glutamate excitotoxicity, oxidative stress, nitric oxide production, calcium overload and inflammation are accompanied[2-3]. Several neuroprotective compounds have been reported to be effective against cerebral ischemia in animal and cell models[4-5]. However, few of these drugs are clinically effective[6]. Thus, new strategies and agents are urgent required for the treatment of ischemic stroke.

Crocin, one of the water-soluble carotenoids, was isolated from *Gardenia jasminoides* and *Crocus sativus*. Crocin exhibits a broad spectrum of pharmacological activities[7-13]. Quite interesting, the neuroprotective effects of crocin against central nervous system (CNS) disorders have also been reported in many *in vitro* and *in vivo* studies[14-15]. Work from Zhang's group disclosed that the pretreatment of crocin alleviated middle cerebral artery occlusion (MCAO) -induced brain injury. It preserved blood brain barrier (BBB) function in the presence of ischemic injury; in the mean time, crocin reduced the loss of tight junction proteins and enhanced NADPH oxidase in the ipsilateral brains of the MCAO-treated rats[16]; While Zheng et al.[17] showed that the pretreatment of crocin markedly inhibited oxidizing reactions and modulated the ultrastructure of cortical microvascular endothelial cells (CMEC) in mice with 20 min of bilateral common carotid artery occlusion (BCCAO) followed by 24 h of reperfusion *in vivo*. These oxidizing reactions including intense superoxide, nitric oxide (NO), and peroxynitrite formed on microvessels and surrounding end-feet may lead to cerebral hemorrhage and edema by disrupting microvascular integrity and breakdown of the BBB.

Of no doubt, the potential therapeutic effects of crocin on ischemic brain injury are of a common sense[18-19]. However, the protective machanism of crocin against focal cerebral ischemic-reperfusion (I/R) injury is far from well addressed. Keeping up with the nitric oxide (NO) excitotoxicity in neuro-damage, we are insterested to disclose whether crocin is capable of reducing NO-induced oxidative stress during ischemic stroke and the mechanism involved. Therefore, in the current investigation, the protection of crocin against rat brain ischemia-reperfusion injury and cerntain aspect of the mechanisms involved were shown here.

1 Materials and methods

1.1 Materials

Crocin was purchased from Chengdu PureChem-Standard Co., Ltd; 3-(4,5-dimethylthiazol-2-yl)－2,5-diphenyl tetrazolium bromide (MTT), Poly-d-lysine, 2,7-dichloro-dihydrofluorescin diacetate (DCFH-DA), caspase-3 activity assay kit, tribromoethanol and triphenyltetrazolium chloride (TTC), and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and horse serum were purchased from Gibco-BRL (NY, USA). Hoechst 33258, BCA protein assay kit were obtained from Beyotime Institute of Biotechnology (Haimen, China); JC-1 dye (MolecularProbes) was from Best-Bio (Shanghai, China). Lactate dehydrogenase (LDH) assay kit was from Jiancheng Biochemical Company (Nanjing, China). Anti-Bcl-2 antibody, anti-Caspase-3 antibody, anti-Bax antibody, anti-cytochrome *c*, anti-β-actin antibodies were purchased from Cell Signaling Technology (China).

1.2 Methods

1.2.1 Experimental animals and treatment Male Sprague-Dawley rats (200 ~ 250 g) were obtained from the Laboratory Animal Centre of Jiangxi University of Traditional Chinese Medicine. Rats were pre-treated with 10, 20, and 40 mg/kg dose of crocin dissolved in saline or vehicle (saline) *via* i. p. injection twice daily for 3 days and the final administration was carried on 30 min before ischemia/reperfusion (I/R) injury. Experiments were approved by the Jiangxi University of Traditional Chinese Medicine Medical Center, Institutional Animal Care and Utilization Committee and were compliant with all regulations.

1.2.2 Rat model of focal middle cerebral artery occlusion Rats were anesthetized with 10% chloral hydrate (300 mg/kg). The middle cerebral artery occlusion (MCAO) model was induced using the intraluminal filament method described previously[20]. Briefly, a midline incision was made on the ventral surface of the neck. Common carotid artery (CCA), internal carotid artery (ICA) and external carotid artery (ECA) were isolated and the ECA was ligated with a suture. An 30 mm polyamide monofilament (diameter 0.24 mm) with a spherical tip was inserted into the ECA and gently advanced about 20 mm to block the blood flow of right middle cerebral artery (MCA). After 2 h occlusion of the MCA, the monofilament was pulled out followed by reperfusion for 24 h. The sham group underwent the same procedure except for monofilament insertion.

1.2.3 Neurological deficit Neurological deficit scores were evaluated at 24 h after reperfusion by two examiners blinded to animal groups, using the modified Bederson's method[21]. The five-point scoring scale was as follows: 0, no observable neurological deficit; 1, mild neurological deficit (forepaw flexion); 2, moderate neurological deficit (forelimb flexion plus decreased resistance to lateral push); 3, severe neurological deficit (unidirectional circling); and 4, very severe neurological deficit (unidirectional circling and decreased level of consciousness).

1.2.4 Infarct size measurement After neurological deficit evaluation, animals were sacrificed following anesthesia with chloral hydrate, and brain tissues were collected quickly. For triphenyl tetrazolium chloride (TTC, Sigma, USA) staining, brain tissues were cut into coronal slices of 2 mm. Brain slices were stained in a 0.1% TTC solution at 37 ℃ for 15 min in dark, and fixed with 4% paraformaldehyde for 24 h. Slices were imaged on each side, and infarct areas in both hemispheres for each slice were measured. Infarct volume was determined as a percentage of the contralateral hemisphere to correct for edema.

1.2.5 Cell culture and treatment PC12 cells were cultured in high-glucose DMEM containing 5% FBS and 10% horse serum, 100 μg of streptomycin/ml, and 100 U of penicillin/mL and incubated at 37 ℃ with 5% CO_2 humidified atmosphere. Cultured media were replaced twice a week with fresh medium as described above. Stock culture was routinely subculture at 1∶5 ratio at a weekly period. For the experiments, cells were pre-incubated with various concentrations of crocin (2.5 ~ 20 μmol/L) for 2 h without other description, and followed by treatment of 750 μmol/L SNP for 24 h.

1.2.6 MTT assay and LDH release assay Cell viability was determined by MTT assay as well as the lactate dihydrogenase (LDH) assay as described in our previous paper[22]. Briefly, after treatment, 20 μL supernatant per well was transferred into a 96-well microplate to determine LDH levels according to the manufacturer's instructions before adding MTT. Optical density was measured using a microplate reader (Bio-Tek, USA) at 405 nm. For the MTT assay, MTT (5 mg/mL) 10 μL was then added to each well and the mixture was incubated for 2 h at 37 ℃. MTT reagent was then replaced with DMSO (100 μL per well) carefully to dissolve formazan crystals. After the mixture was shaken at room temperature for 10 min, absorbance was determined at 570 nm using a microplate reader (Bio-Tek, USA). Results were expressed as the percentage of the absorbance of control cells, which was set as 100%.

1.2.7 Morphologic changes PC12 cells grown on 48 – well plates were treated with crocin and/or SNP as described above. After that, cells were fixed with 4% paraformaldehyde and stained with Hoechst 33258 (5 μg/mL) for 10 min at 37 ℃ in the dark. Then Hoechst 33258 was removed by washing with PBS, and morphologic changes were observed by phase-contrast

microscopy and cells images were taken using a fluorescence microscope (IX71, Olympus, Tokyo, Japan).

1.2.8 **Measurement of ROS** Intracellular ROS formation was measured by fluorescence using DCFH-DA. Briefly, after treatment, cells were washed and then stained with 10 μmol/L DCFH-DA in serum-free medium for 30 min at 37 ℃ in the dark. The fluorescence from the DCF was analyzed using a fluorescence plate reader (Flex Station3, Molecular Devices, USA) at excitation and emission wavelengths of 488 and 525 nm, respectively, and taken images using a fluorescence microscope (IX71, Olympus, Tokyo, Japan).

1.2.9 **MMP determination** MMP was analyzed by using a fluorescent dye JC-1 (BestBio Shanghai China). JC-1 penetrates cells and healthy mitochondria. At low membrane potentials (apoptotic cells), JC-1 exists as a monomer which emits green fluorescence. JC-1 aggregates and emits red fluorescence at higher membrane potentials (non-apoptotic cells). Assays were initiated by incubating PC12 cells with JC-1 (5 mg/L) for 20 min at 37 ℃ in the dark and the fluorescence of separated cells were captured using inverted fluorescence microscopy (Olympus, Japan, at wavelengths of 490 nm excitation and 530 nm emission for green, and at 540 nm excitation and 590 nm emission for red). The ratios of red/green fluorescence were calculated.

1.2.10 **Western blotting analysis** Western blotting analysis was performed as previously described[23]. Briefly, cells from different experimental conditions or the hippocampus were lysed with ice-cold RIPA lysis buffer, and protein concentration was determined with a BCA protein assay kit according to the manufacturer's instructions. Equal amounts of lysate protein (20 μg/lane) were subjected to SDS-PAGE with 10% polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. After transfer nitrocellulose blots were first blocked with 3% bovine serum albumin (BSA) in PBST buffer (PBS with 0.01% Tween 20, PH 7.4), and incubated overnight at 4 ℃ with primary antibodies in PBST containing 1% BSA. Immunoreactivity was measured by sequential incubation with horseradish peroxidase-conjugated secondary antibodies, and detected by the enhanced chemiluminescence technique.

1.2.11 **Statistical analysis** Data are expressed as the mean ± SEM for 3 ~ 5 experiments. The statistical significance of differences between the mean values for the treatment groups was analyzed with one-way or two-way analysis of Variance (ANOVA) followed by Dunnet *t*-tests using the software SPSS 13.0 (Chicago, USA). $P < 0.05$ was considered statistically significant.

2 Results

2.1 Crocin attenuated focal cerebral ischemic/reperfusion injury in rats

In order to evaluate the protective effects of crocin against I/R injury in rats, the *in vivo* experiments were carried on. The rats were pre-treated with crocin at a dose of 10, 20, 40 mg/kg twice a day for 3 days and a 2 h MCAO followed by a 24 h reperfusion was applied on the rats to induce a cerebral ischemia injury model. The neurological deficit scores were assessed at 24 h after reperfusion, then the animals were sacrificed. Infarct volumes were determined by 2, 3, 5-triphenyltetrazolium chlorid (TTC) staining. The results were listed in Table 1 and Fig. 1. It can be seen from Table 1 that no neurological deficits were observed in sham-treated rats, while MCAO-processed rats showed high scores (2.22 ± 0.74) in neurological assessment. As expected, crocin reversed the score at the dose of 20 (score = 1.40 ± 0.33), and 40 (score = 1.31 ± 0.47) mg/kg, respectively. TTC staining of brain sections showed that crocin decreased infarct volume in a dose-dependent manner in MCAO rats (Fig. 1A-B), in which the infarct volume dropped from 25.2% in vehicle group to 15.1% in 20 mg/kg crocin-treated group, and 13.6% in 40 mg/kg crocin-treated group. These results suggested that crocin-pretreatment markedly improved the neurological dysfunction and protected ras against ischemic brain injury.

Table 1 Effect of crocin on neurological deficit[1)]

Group	Dose	*n*	Neurologic score
Sham	Saline	10	0
Vehicle	Saline	10	2.22 ± 0.74#
Crocin	10 mg/kg	10	1.75 ± 0.41
Crocin	20 mg/kg	10	1.40 ± 0.33 *
Crocin	40 mg/kg	10	1.31 ± 0.47 *

1) Values are mean ± SD. # $P < 0.05$ vs sham group; * $P < 0.05$ vs vehicle group.

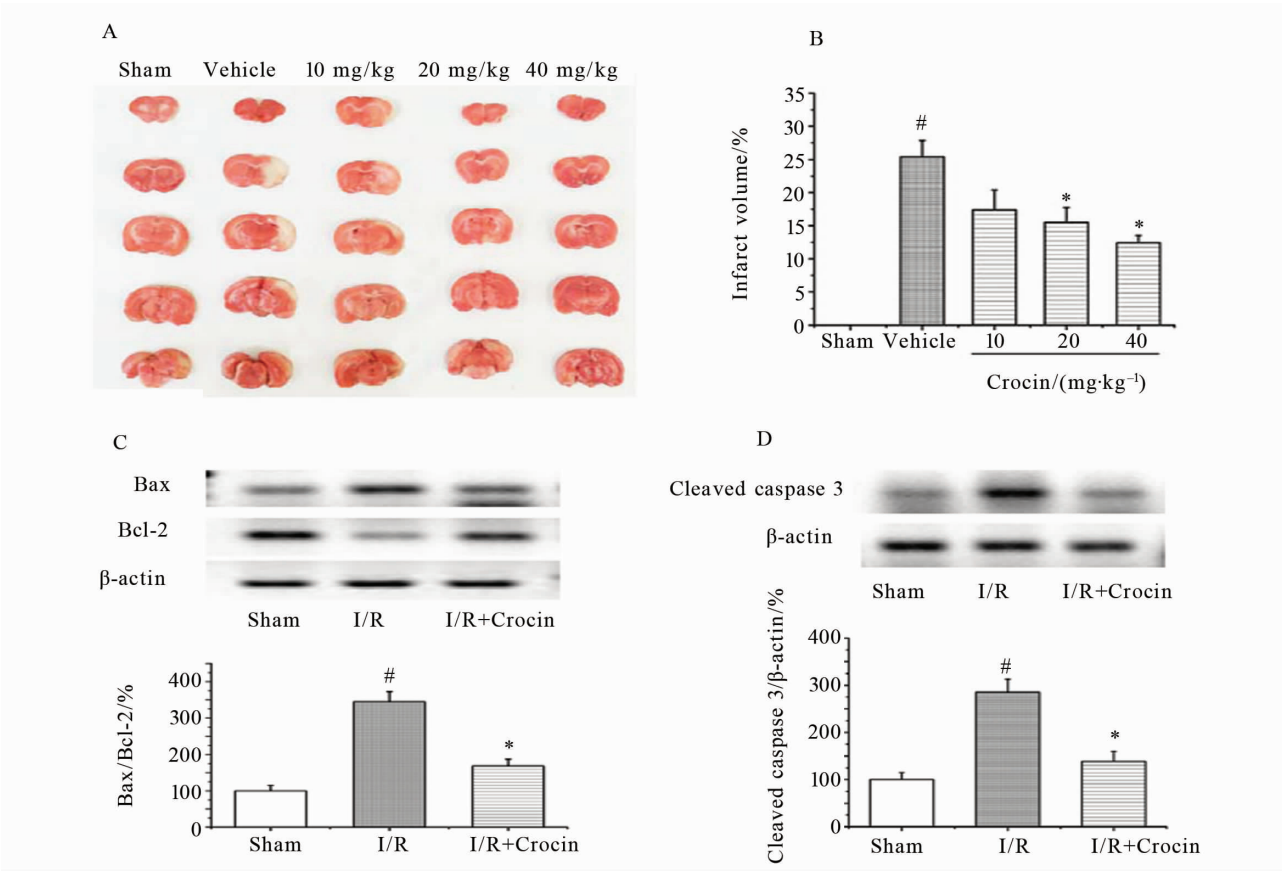

Fig. 1 Effects of Crocin on infarct volume and expression of apoptosis-related proteins in hippocampus of MCAO rats (A) Infarct areas and volume ratios were assessed by TTC staining; (B) Infarct volume ratios in each group. Bars represent mean ± SD of 5 brains. $^{\#}P < 0.05$ versus Sham group; $*P < 0.05$ versus Vehicle group; (C) The representative western blot image of Bax/Bcl-2 protein (upper) and semi-quantitative analysis of Bax/Bcl-2 content (lower) from various groups , Sham: Sham group; I/R: I/R group; I/R + Crocin: I/R + Crocin (30 mg/kg) group; (D) The representative western-blot image of cleaved caspase-3 protein (upper) and semi-quantitative analysis of cleaved caspase-3 content (lower) in different groups. Sham: Sham group; I/R: I/R group; I/R + Crocin: I/R + Crocin (30 mg/kg) group. Values are mean ± SEM ($n = 5$), $^{\#}P < 0.05$ versus Sham group, $*P < 0.05$ versus I/R group.

2.2 Effects of crocin on the expression of Bax, Bcl-2 and cleaved caspase-3 in hippocampus of MCAO rats

To determine whether crocin administration leads to changes in B-cell lymphoma 2 (Bcl-2), Bcl-2 associated protein X (Bax), and cleaved caspase-3 protein levels in hippocampus of cerebral I/R rats, we examined the expression of these proteins in hippocampal from rats in three groups (Sham: Sham group; I/R: I/R group; I/R + Crocin: I/R + Crocin (30 mg/kg) group.) by western blotting analysis. As shown in Fig. 1C-D, expression of pro-apoptotic Bax in hippocampus was much higher in I/R group than that in sham group. In contrast, crocin administration resulted in a significant decrease in Bax protein expression (Fig. 1C). On the other hand, I/R injury caused a noticeable decrease of Bcl-2 compared to sham group; while crocin pretreatment significantly up-regulated Bcl-2 level in hippocampus. Thus the calculated Bax/Bcl-2 ratio for the hippocampus was higher in the I/R group than that in the sham group, which was 350%; while crocin markedly reduced Bax/Bcl-2 ratio in hippocampus of the I/R group, which was 175%. Caspase-3 cleavage is down-stream of the Bcl-2/Bax family apoptotic cascade and may integrate apoptotic signaling from different pathways as well. Low levels of cleaved Caspase-3 were detected in the hippocampus of sham group animals but its level was dramatically increased in I/R group animals. And crocin administration significantly diminished cleaved caspase-3 protein

expression (Fig. 1D).

2.3 Protective effect of Crocin in the PC12 cells against SNP injury

NO excitotoxicity is thought to be one of key neurotoxic mechanisms in cerebral ischemia-reperfusion. Zheng et al. confirmed that transient global cerebral ischemia (20 min), followed by 24 h of reperfusion, significantly promoted generation of NO and malondialdehyde (MDA) in cortical microvascular homogenates[17]. In order to evaluate how crocin prevent NO excitotoxicity, a cell line derived from a pheochromocytoma of the rat adrenal medulla (PC12) was applied because of cheap and convenience. Sodium Nitroprusside (SNP) was used to set up NO excitotoxic PC12 cell model[24-25].

As shown in Fig. 2A, treatment of SNP for 24 h significantly decreased the cell viability of PC12 cells in a concentration-dependent manner as compared to the control group. The toxicity of SNP at 750 μmol/L caused about 38% decrease in cell viability. And therefore this concentration was used in further experiments to generate NO insult. To find out the concentrations of crocine that do not induce cell toxicity, PC12 cells were treated with various concentrations of crocin for 24 h and then cell viability and cytotoxicity were monitored by MTT assay. No significant difference was found among groups treated without or with crocin at a dose of 2.5 to 40 μmol/L (Fig. 2B). To investigate the neuroprotective effects of crocin against SNP-induced oxidative damage, PC12 cells pretreated with various concentrations of crocin were incubated with SNP for 24 h, the viability of cells was determined by

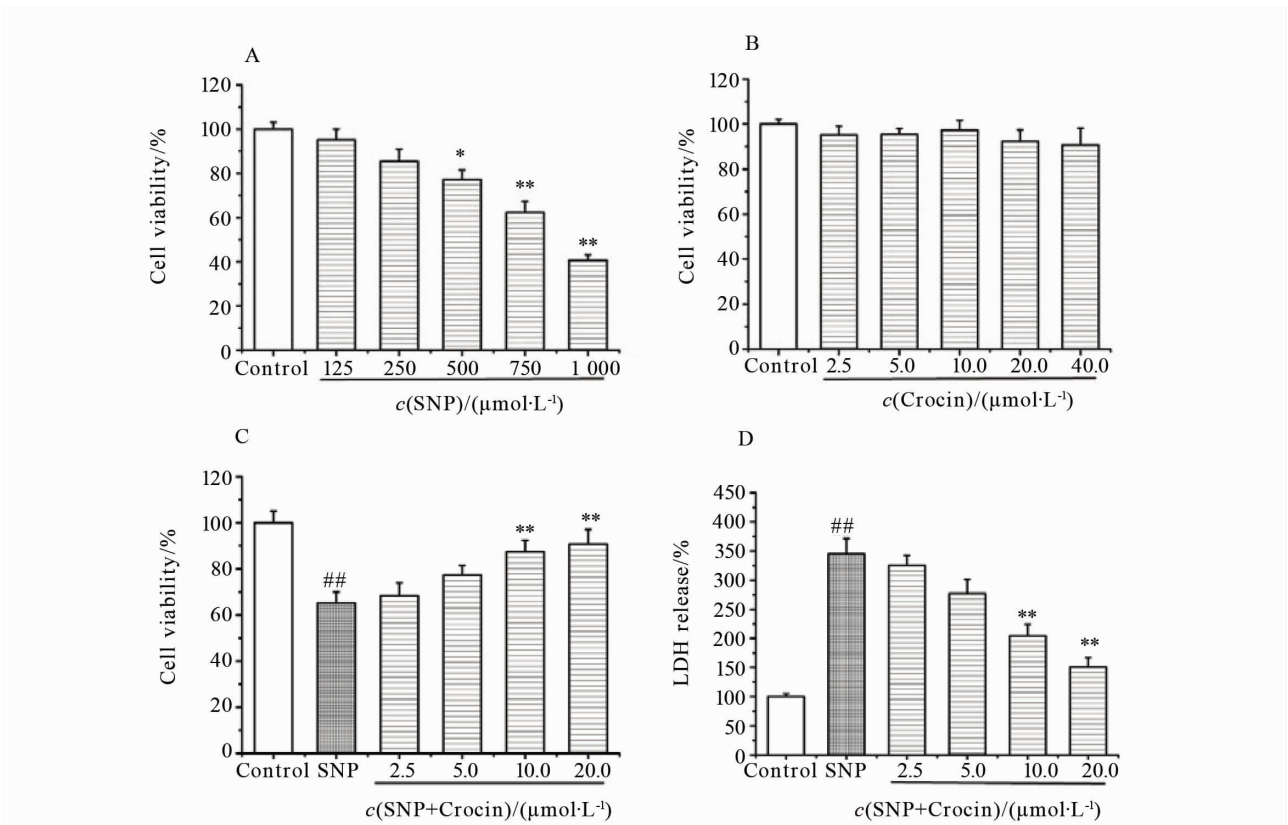

Fig. 2 Neuroprotection of PC12 cells by crocin against excitotoxicity induced by SNP (750 μmol/L)

(A) The cytotoxicity of SNP on PC12 cells. Cells were treated with SNP (125 ~ 1 000 μmol/L) for 24h and cell viability was measured using MTT assay. $*P<0.05$, $**P<0.01$ versuscontrol group; (B) Effects of crocin on the cell viability of PC12 cells. PC12 cells were treated with Crocin (2.5 ~ 40 μmol/L) for 24 h. Cells viability were determined by MTT assay. $*P<0.05$, $**P<0.01$ versus control group; (C) PC12 cells were treated with different concentration of crocin (2.5 ~ 20 μmol/L) for 2 h and then incubated with 750 μmol/L SNP for a further 24 h. Cells viability were determined by MTT assay; (D) LDH results. $^{\#\#}P<0.01$ versus control group; $*P<0.05$, $**P<0.01$ versus SNP-treated group ($n = 6$).

MTT assay. As indicated in Fig. 2C, crocin dose-dependently decreased SNP-caused cytotoxicity, at the concentrations from 2.5 μmol/L to 20 μmol/L. Crocin reversed SNP insults by 38.7% at a dose of 20 μmol/L. Moreover, the effects of crocin on SNP-induced LDH release was also studied. The results showed that treatment of SNP alone significantly induced LDH release which was markedly reversed by 10 ~ 20 μmol/L of crocin pretreatment (Fig. 2D).

To determine whether crocin blocks SNP-induced apoptosis, DNA staining with Hoechst 33258 was used to evaluate the nuclear condensation. As shown in Fig. 3A, SNP induced cell apoptosis in PC12 cells, which was characterized by nuclear condensation and the presence of apoptotic bodies. In contrast, pre-incubation of PC12 cells with 10 μmol/L of crocin significantly decreased SNP-induced apoptotic cells (Fig. 3B). The apoptosis rate was dropped down from 35% (SNP) to 12% (SNP + Crocin).

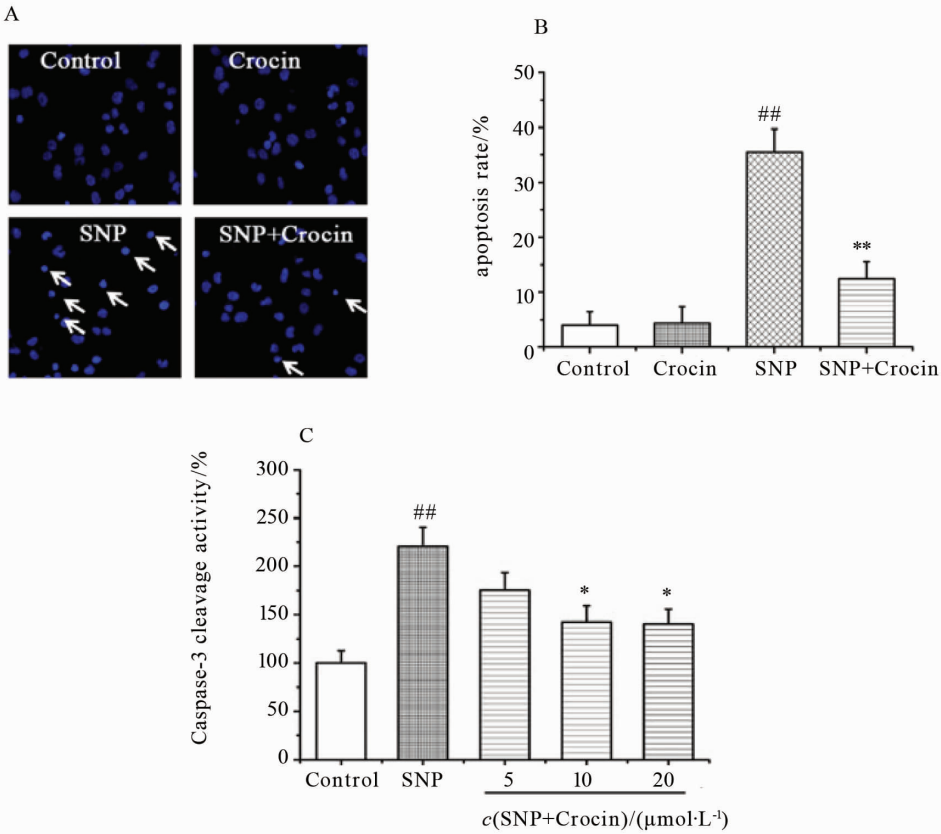

Fig. 3　Crocin protected PC12 cells from SNP-induced apoptosis

PC12 cells were pretreated without or with 10 μmol/L of crocin for 2 h and then treated with or without 750 μmol/L SNP for 24 h. (A) Crocin significantly attenuated SNP-induced morphologic changes and nuclear condensation. Representative images were taken by a fluorescence microscope. The images shown are representative of three experiments; (B) Histogram showing the percentage of apoptosis in PC12 cells. Cells were stained with Hoechst 33258, and apoptosis was detected by a high-content screening system. The proportion of apoptosis (%) was determined as the number of apoptotic cells to total number of cells; (C) Cells were pretreated with or without Crocin (5 ~ 20 μmol/L) for 2 h and then cultured in the presence or absence of 750 μmol/L SNP for 12 h. Activities of Caspase-3 were measured by a fluorometric method. $^{\#\#}P < 0.01$ versus control group; $*P < 0.05$, $**P < 0.01$ versus SNP-treated group ($n = 6$).

Similarly to the case in the hippocampus of I/R rats, SNP increased the activity of cleaved caspase-3 by 2.25 times compared to that of control group (Fig. 3C); while crocin dose-dependently reversed the up-regulated activity of caspase-3 by SNP.

2.4 Effects of Crocin on SNP-induced ROS production and mitochondrial membrane potential (MMP) collapse

Previous studies have shown that the toxicity of SNP was mediated through the production of ROS[26]. Therefore, we investigated whether crocin down-regulates SNP-induced ROS accumulation in PC12 cells. Cellular ROS was determined by DCFH-DA staining, which is a ROS probe[23]. In this experiment, PC12 cells were pretreated without or with 10 μmol/L crocin for 2 h and then treated without or with 750 μmol/L SNP for 24 h. Indicated in Fig. 4A-B, the microscopic images showed that SNP treatment increased the intracellular production of ROS by 2.5 times compared to that of control group. Treatment of 10 μmol/L crocin did not raise the ROS level; while crocin pretreatment attenuated the up-regulated ROS level by SNP, which was decreased by 40%.

We also measured the MMP affected by SNP with/without crocin treatments. In this experiment, PC12 cells were incubated with JC-1 (5 mg/L) for 20 min at 37 ℃ in dark and MMP assay was performed. The shift of fluorescence from red to green indicated by JC-1 reflected the decline of the membrane potential and early apoptosis. It can be seen from Fig. 4C-D that 10 μmol/L crocin treatment alone did not cause the decline of MMP in PC12 cells; while 750 μmol/L treatment of SNP significantly induced the decline of MMP, in which it was decreased by 37.5% compared to that of control group. Quite interestingly, pre-treatment of cells with 10 μmol/L crocin significantly reversed the decline of MMP induced by SNP, which the MMP was reversed by 40% (Fig. 4D).

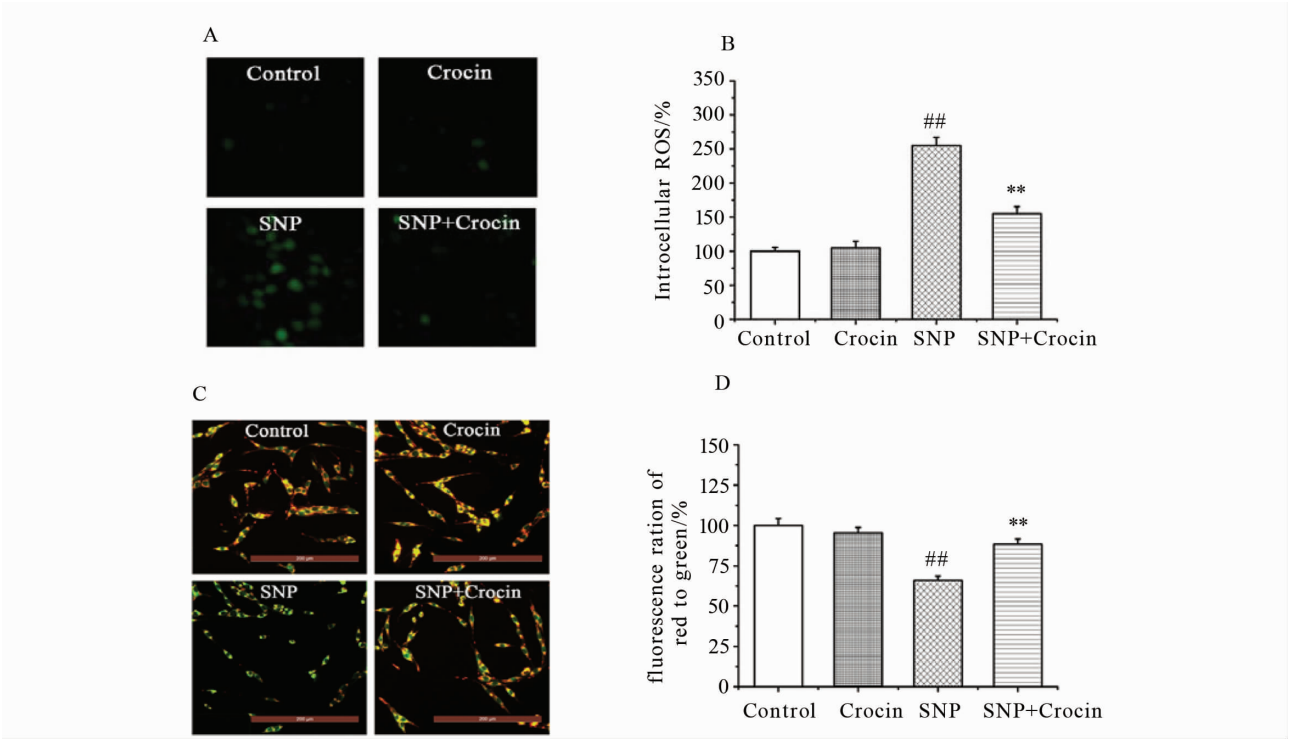

Fig. 4 Effects of Crocin on the intracellular ROS and the reduction of MMP induced by SNP insult in PC12 cells

(A) PC12 cells were pretreated without or with 10 μmol/L of crocin for 2 h and then treated with or without 750 μmol/L SNP for 12 h. The fluorescence intensity of DCFH-DA showed that Crocin blocked the ROS accumulation induced by SNP; (B) Histogram showing the ROS level in PC12 cells after expose to SNP in presence or absence of crocin compared to control groups. Intracellular ROS levels (%) were expressed as the mean intensity of fluorescence (MIF) within the live cell and were measured by a high-content screening system after cells were stained with DCFH-DA; (C) MMP was determined as described in Materials and Methods. SNP insult caused the decline of MMP in PC12 cells. Crocin reversed the effects of SNP insult; (D) Quantitative data of the Red/Green ratio. $^{\#\#}P < 0.01$ versus control group; $**P < 0.01$ versus SNP-treated group ($n = 6$).

2.5 Effects of crocin on expression of Bcl-2/Bax and the release of cytochrome *c* in SNP-treated PC12 cells

It is well known that the Bax/Bcl-2 ratio plays an essential role during apoptosis. I/R injury in rats increased Bax/Bcl-2 ratio in the hippocampus (Fig. 1C). To our expect, 750 μmol/L SNP treatment increased the expression of Bax, while in the meantime decreased the expression of Bcl-2 (Fig. 5A-B), whereas the Bax/Bcl-2 ration was increased by 2.65 times compared to that of control group. However, pretreatment with crocin reversed the effect of SNP dose dependently. Pretreatment of 5, 10 and 20 μmol/L crocin altered the Bax/Bcl-2 ratio by 32.9%, 45.5% and 52.2%, respectively, as compared to that of the SNP-treated group.

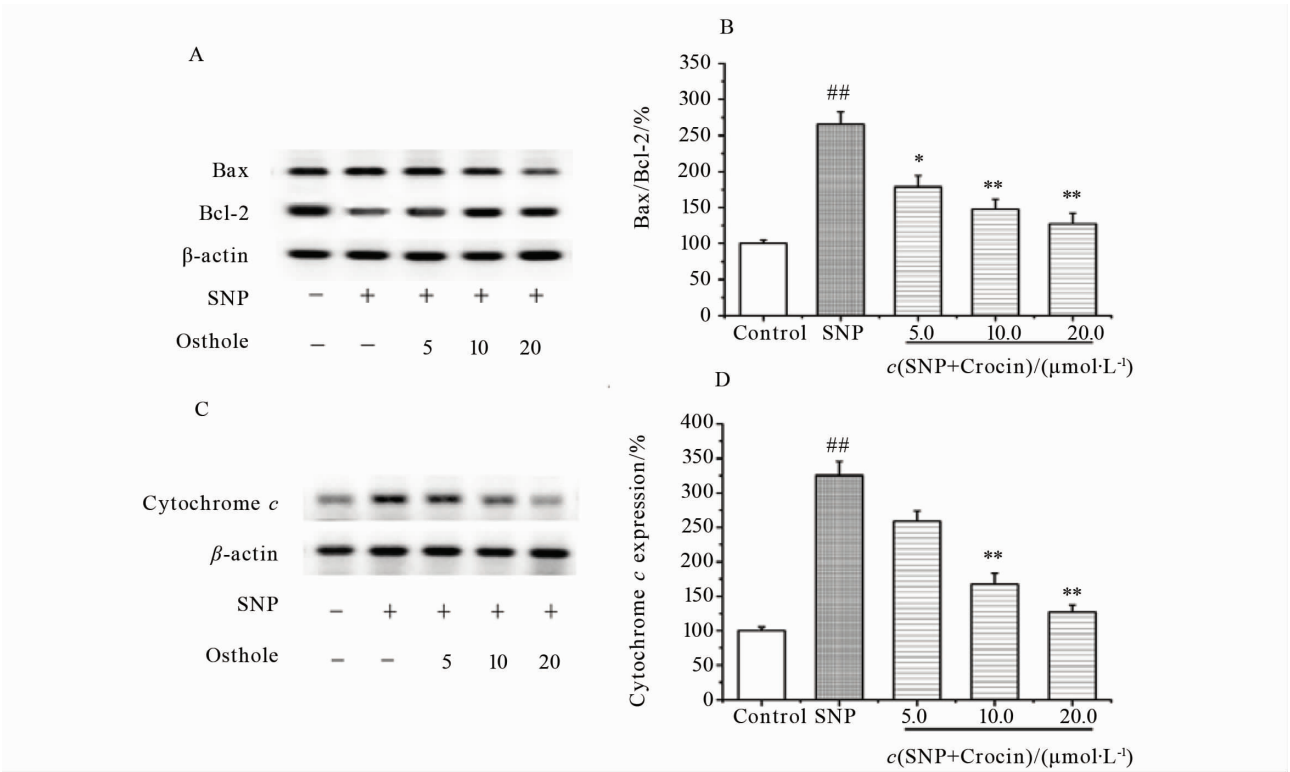

Fig. 5　Crocin inhibited SNP-induced mitochondrial apoptotic pathway in PC12 cells

Cells were pretreated with or without crocin (5 ~ 20 μmol/L) for 2 h and then cultured in the presence or absence of 750 μmol/L SNP for 12 h. Cell lysates were subjected to Western blot analysis. (A) Crocin prevented SNP-induced changes in Bcl-2 family member expression; (B) The levels of Bax and Bcl-2 were quantified by densitometric analysis and the Bax/Bcl-2 ratio was determined; (C) Crocin inhibited SNP-induced mitochondrial release of cytochrome *c*; (D) The amount of cytochrome *c* was estimated by densitometric analysis of each protein band. The data were represented as means ± SD for three independent experiments. Equal protein loading was confirmed by analysis of β-actin in the protein extracts. $^{\#\#}P < 0.01$ versus control group; $*P < 0.05$, $**P < 0.01$ versus SNP-treated group ($n = 3$).

The mitochondrial permeability transition pore opening is associated with the collapse of the membrane voltage, and leads to the release of cytochrome c into the cytosol. Cytochrome c release has been shown to play a critical role in cell apoptosis[27]. Therefore, using Western blotting, we investigated the possible effect of crocin on SNP-induced cytochrome c release from mitochondria. As shown in Fig. 5C-D, SNP caused the significant cytochrome c release, which was 3.3-fold of the control. However, the induction was markedly inhibited in the presence of crocin. The degree of inhibition increased from 48.6% to 61% when the dose of crocin increased from 10 to 20 μmol/L (Fig. 5D).

3 Discussion

There are cumulative evidences suggesting the involvement of reoxygenation during experimental ischemia and reperfusion models, such as transient focal/global ischemia in rodents[28]. Intense superoxide, NO, and peroxynitrite formation on microvessels and surrounding end-feet may lead to cerebral hemorrhage and edema by disrupting microvascular integrity and breakdown of the BBB. Agents which prevent ischemic reperfusion injuries in cerebral microvessels and diminishes oxidative/nitrative stress may reduce reperfusion-induced injury and may extend the therapeutic targets[29-30].

It was reported that NO content and nitric oxide synthase (NOs) activities in cortical microvascular homogenates were significantly increased in transient global ischemia mice. And oral administration of crocin (20, 10 mg/kg) significantly inhibited the increased NO content and NOS activities[17]. We confirmed this protective effect by applying the SNP-impaired PC12 cell model, where crocin reversed SNP insults by 38.7%.

As we know, antioxidant therapy is an attractive strategy against neuronal loss In neurodegenerative diseases[31-32]. Based on it's chemical structure, crocin is a no doubt an antioxidant. This is the reason why crocin showed neuroprotective effects against CNS disorder. Lee et al. believed that the antioxidant effects of crocin is one of the mechanism against transient global cerebral ischemia[33]. 750 μmol/L SNP treatment induced oxidative/nitrative stress, crocin may lower the stress level.

Pathological ROS may affect mitochondrial membrane and cause apoptosis[34]. In the current study, crocin attenuated accumulation of intracellular ROS in PC12 cells. We observed that SNP insult was followed by the loss of the MMP, which was significantly reversed by crocin. Previously we had reported that NO production in PC12 cells induced apoptosis via caspase-3 activation[25]. It was found that SNP did increase the activity of caspase-3 in PC12 cells, which could be reversed by crocin. Undoubtedly, caspase-3 was involved in the apoptotic process, where it is responsible for chromatin condensation and DNA fragmentation[35].

Bcl-2 family proteins and the ratio of the Bax/Bcl-2 regulate apoptosis in the intrinsic apoptotic pathway[36]. The present study showed that SNP could up-regulate the expression of Bax and down-regulate Bcl-2 with an increase in Bax/Bcl-2 ratio, which could be reversed by crocin co-treatment. Bcl-2 stabilizes membrane permeability, preserves mitochondria integrity, and suppresses the release of Cytochrome c[37]. As shown in Fig. 4, SNP promoted cell apoptosis by releasing Cytochrome c from the mitochondrial inner space to cytosol which was reversed by crocin. All these findings indicated that crocin exerts protective effects against SNP-induced apoptosis, partly through the attenuation of the mitochondrial apoptotic pathway. The I/R injury also increased Bax/Bcl-2 ratio, and crocin lowered this ratio. Therefore, we believe this is one possible mechanisms which crocin protect against I/R insults in rats.

Ischemic insults cumulate to neuron death, a manifestation that is particularly prominent in the hippocampus carbonic anhydrase 1 (CA1) region in which the neurons are susceptible to I/R injury[38]. In accordance with other studies, the present study demonstrated that cerebral I/R induced extensive hippocampus CA1 neuron injury 24 h after reperfusion, as evidenced by an increase in the expression of Bax and cleaved Caspase-3 and an increase of Bax/Bcl2 ratio, implying apoptosis as an important contributor to I/R-induced neuron death. Of notice, these alterations were significantly attenuated by treatment with crocin, suggesting the anti-apoptotic potential of Crocin.

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