

Effects of crocin on brain oxidative damage and aversive memory in a 6-OHDA model of Parkinson's disease

Efeitos da crocina no dano oxidativo cerebral e na memória aversiva em um modelo 6-OHDA de doença de Parkinson

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ABSTRACT

The purpose of the present study was to investigate the effect of crocin on brain oxidative damage and memory deficits in a 6-hydroxydopamine (6-OHDA) model of Parkinson's disease. Male Wistar rats were subjected to unilateral injection of 6-OHDA (16 µg) into the medial forebrain bundle and treated with crocin (30 and 60 mg/kg) for six weeks. The rats were tested for memory performance at six weeks after 6-OHDA infusion, and then were killed for the estimation of biochemical parameters. The increase in thiobarbituric acid reactive substances (TBARS) and nitrite levels in the hippocampus were observed in the 6-OHDA lesioned rats, which was accompanied by memory deficits in a passive avoidance test at the end of week 6. Moreover, treatment with crocin decreased TBARS and nitrite levels in the hippocampus, and improved aversive memory. The present study conclusively demonstrated that crocin acts as an antioxidant and anti-inflammatory agent in the hippocampus of parkinsonian rats and could improve aversive memory through its properties.

Keywords: Crocin; 6-hydroxydopamine; oxidative stress; nitric oxide; aversive memory; medial forebrain bundle.

RESUMO

O objetivo do presente estudo foi investigar o efeito da crocina no dano oxidativo cerebral e nos déficits de memória em um modelo 6-OHDA de doença de Parkinson. Ratos Wistar machos foram submetidos à injeção unilateral de 6-OHDA (16 µg) em MFB e tratados com crocina (30 e 60 mg/kg), durante 6 semanas. Os ratos foram testados quanto ao desempenho da memória 6 semanas após a infusão de 6-OHDA, e, em seguida, foram sacrificados para a estimativa dos parâmetros bioquímicos. O aumento nos níveis de TBARS e de nitrito no hipocampo foram observados em ratos 6-OHDA lesionados, acompanhado por déficits de memória em um teste de esquiava passiva no final da semana 6. Além disso, o tratamento com crocina diminuiu os níveis de nitrito e de TBARS no hipocampo e melhorou a memória aversiva. O presente estudo demonstrou conclusivamente que a crocina age como um antioxidante e um agente anti-inflamatório no hipocampo de ratos parkinsonianos e pode melhorar a memória aversiva através de suas propriedades.

Palavras-chave: Crocin; 6-hidroxidopamina; estresse oxidativo; óxido nítrico; feixe medial do cérebro anterior.

Parkinson's disease (PD) is a progressive neurodegenerative disorder that is characterized by the degeneration of dopaminergic nigrostriatal neurons, which leads to motor symptoms of bradykinesia, rigidity, rest tremor, and postural imbalance¹. Parkinson's disease also causes impairments in cognitive performance, and the progression of these deficits can lead to dementia². The hippocampus, which is involved in cognitive processes such as learning and memory, is implicated in memory deficits observed in PD since both structural and functional changes of the

hippocampus have been observed in PD patients^{3,4}. Several MRI studies have also clearly revealed that the reduction of hippocampal volume was accompanied by cognitive deficits in PD patients⁵.

Numerous studies have suggested that oxidative stress plays a major role in the pathogenesis of PD⁶. Free radicals and other reactive oxygen species (ROS) resulted from dopamine auto-oxidation and metabolism, lipid peroxidation, impaired mitochondrial function, and deficiencies in endogenous antioxidant systems that may all contribute

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to a progressive loss of dopaminergic neurons^{7,8}. There is also some evidence showing that the neurotoxicity of 6-hydroxydopamine (6-OHDA), for the modeling of PD, is due to its oxidation and the formation of various oxidants and free radicals and the depletion of reduced glutathione⁹. This leads to lipid peroxidation, protein damage and ultimately degeneration of the nigrostriatal dopaminergic system. 6-OHDA has also been reported to produce reactive nitrogen species (RNS) such as nitric oxide (NO) by elevated expression of inducible nitric oxide synthase (iNOS), especially in neurons¹⁰. Reactive oxygen species can rapidly interact with NO and subsequently produce more powerful oxidant peroxynitrite. Peroxynitrite is known to structurally and functionally modify critical cellular macromolecules and cause oxidative damages, which finally leads to apoptotic cell death¹¹.

Recently, the major focus of many preclinical studies is the identification of drugs or approaches that might prevent or inhibit the neurodegenerative process. In this context, it has been suggested that antioxidant molecules (carotenoids and polyphenols) and compounds interfering with production of reactive oxygen species and nitric oxide might be protective.

Crocin is a water-soluble carotenoid and an active constituent of saffron (*Crocus sativus* L.). It has been reported that crocin possesses multiple pharmacological properties, including antioxidative activity^{12,13}, anti-inflammatory¹⁴, protection against cardiovascular diseases, inhibition of tumor cell proliferation, neuroprotection and protection of hepatocytes¹². It has also been shown that the spice saffron, which contains powerful antioxidants such as crocin, protects nigral and retinal dopaminergic cells in an acute MPTP mouse model of Parkinson's disease¹⁵. The antioxidant and radical scavenging activity of crocins have also been shown in several *in vitro* models^{12,16}. Based on the role of oxidative and nitrosative stress on the pathophysiology of PD and considering the antioxidant and anti-inflammatory effects of crocin, the present study was designed to evaluate whether it protects against 6-OHDA-induced oxidative damage and memory deficits in rats.

METHODS

Animals

Adult male Wistar rats, weighing 250–300g were housed in an air conditioned colony room at 22°C ± 2°C on a standard pellet diet and tap water *ad libitum*. The Ethics Committee for Animal Experiments at Isfahan University of Medical Sciences approved the study and all experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications N° 8023, revised 1996).

Chemicals

Crocin, 6-OHDA, and apomorphine hydrochloride were purchased from Sigma Aldrich Co. 2,2'-Dinitro-5,5'-dithiodibenzoic acid (DTNB), trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA), Tris-EDTA, chloral hydrate and hydrochloric acid (HCL) were obtained from Merck.

Experimental design

The animals were randomly divided into four groups, with eight rats in each group, as follows: normal saline sham-operated group, normal saline-treated lesioned group (6-OHDA), and the crocin-treated lesioned groups (crocin 30 and 60 mg/kg/day). Crocin was dissolved in normal saline and injected at doses of 30 and 60 mg/kg/day intraperitoneally, three days before the surgery for six weeks. The surgical procedures were carried out under general (chloral hydrate, 450 mg/kg, ip) and local anaesthesia (lidocaine 2%). The rats were placed in a stereotaxic apparatus and the lesion was induced by injection of 6-OHDA (16 µg/4µl 0.2% ascorbate saline) into the left medial forebrain bundle through microinjection pump (Kd Scientific, USA) according to the coordinates: AP: -4.5 mm; ML: -1.7 mm; DV: -8.2 mm¹⁷. The rats of the sham-operated group also received an identical volume of the ascorbate saline as the vehicle. The injection rate was 1 µl/min and the needle was kept in place for an additional five minutes before slowly being retracted. At the end of the experiment, the animals were sacrificed and the hippocampus and cortex were dissected out, washed immediately in ice-cold saline, and homogenized in the NaCl solution by a homogenizer. The hippocampus and cortex were obtained bilaterally, and the data are measures from combined bilateral tissues.

Lipid peroxidation levels

The lipid peroxidation level of the hippocampus and cortex was measured as malondialdehyde, which is the end product of lipid peroxidation. The malondialdehyde reacts with TBA as a thiobarbituric acid reactive substance (TBARS) and produces a red colored complex that has a peak absorbance (A) at 535 nm. Briefly, a mixture of TCA, TBA, and HCl were added to 1mL of homogenate, and the mixture was heated for 45 min in a boiling water bath. After cooling, the samples were centrifuged at 1000× g for 10 min and the absorbance was measured at 535 nm. The level of TBARS was calculated according to follow equation¹³. Concentration (Molar) = Absorbance / 1.65 × 10⁵.

Total thiol concentration

Total sulfhydryl (SH) groups were measured using DTNB as the reagent. This reagent reacts with the SH groups to produce a yellow colored complex that has a peak absorbance at 412 nm. Briefly, 1mL Tris-EDTA buffer (pH = 8.6) was added to 50 µL homogenate in 2 mL cuvettes and the sample absorbance was read at 412 nm

against the Tris-EDTA buffer alone (A1). Then, 20 μL of the DTNB reagent (10 mM in methanol) was added to the mixture and after 15 min, the sample absorbance was read again (A2). The absorbance of the DTNB reagent was also read as a blank (B). The total thiol concentration (mM) was calculated by: The total thiol concentration (mM) = $(A2-A1-B) \times 1.07/0.05 \times 13.6^{13}$.

Glutathione peroxidase assay

GSH peroxidase concentration was measured with the GSH peroxidase kit (Randox Labs, Crumlin, UK).

Nitrite levels

The level of nitrite was measured using a colorimetric assay kit (Promega Corporation, USA) that involved the Griess reaction. Briefly, after adding sulphanilamide solution and incubation, N-(1-naphthyl) ethylenediamine solution was added. Then, the sample absorbance was measured by a microreader in the wavelength of 492 nm. The nitrite concentration of samples was determined by comparing comparison against with the nitrite standard reference curve.

Passive avoidance learning

The passive avoidance test was used in the current study, because it is a suitable model for evaluating hippocampal-dependent memory deficits in experimental animals. Passive avoidance learning was assessed by shuttle box at the end of week 6. The apparatus consisted of a light compartment (25 \times 25 \times 20 cm) and a dark compartment (50 \times 25 \times 20 cm) with a grid floor and wooden walls. The two compartments were separated by a sliding guillotine door. On the day before training, each rat was placed into the apparatus and allowed to move around 5 min for five minutes for habituation. In the training session, animals were placed individually in the light compartment for one minute. After opening the door and entrance of the rat entering into the dark chamber, the door was closed and a 1mA foot electric shock was delivered for 3s through the grid floor. The current intensity for foot shock was selected based on previous studies in our laboratory. In the test session, each rat was again placed into the light compartment. The step-through latency to enter the dark compartment was measured as a positive index of memory performance, with a 300 s cut-off time¹⁸.

Histology

The animals were sacrificed by a high dose of the anaesthetic. Then, the brains were removed and stored in 10% formalin for 72 h. The brains were sectioned coronally at 40 μm by a freezing microtome (Leica, Germany). Sections were mounted on gelatin-coated slides and studied using a light microscope. The track of the needle and injection site of 6-OHDA (Figure 1) was determined by reference to a rat brain atlas¹⁷.



mt: mammillothalamic tract, F: fornix.

Figure 1. Photograph of the coronal section of rat brain representing injection site of 6-OHDA in the medial forebrain bundle (open circle).

Statistical analysis

The data were expressed as mean \pm SEM. Statistical analysis was carried out using one-way ANOVA followed by the LSD post hoc test. A statistical p-value < 0.05 was considered significant.

RESULTS

Effects of crocin on lipid peroxidation levels

As shown in Figure 2, a significant increase in the levels of TBARS, an index of lipid peroxidation, was found in the hippocampus of 6-OHDA-lesioned rats ($p = 0.01$) as compared with the sham group. Moreover, treatment of lesioned rats with crocin at a dose of 60 mg/kg reduced the TBARS levels in the hippocampus at the end of week 6 ($p < 0.05$, Figure 2). Meanwhile, 6-OHDA increased the TBARS levels in the cortex of lesioned rats, although the changes were not significant.

Effects of crocin on nitrite levels

Figure 3 shows the nitrite levels in the hippocampus and cortex of the sham and experimental groups. A significant increase in the nitrite levels in the hippocampus ($p < 0.05$) of 6-OHDA-lesioned rats was observed as when compared with sham group rats. Treatment of 6-OHDA-lesioned rats with crocin at a dose of 30 mg/kg significantly decreased the nitrite levels in the hippocampus as when compared to with the lesioned group ($p < 0.05$, Figure 3). Meanwhile, 6-OHDA

did not change the nitrite levels in the cortex of lesioned rats compared with sham group rats (Figure 3).

Effects of crocin on total thiol concentration

Figure 4 shows the total thiol concentration in the hippocampus and cortex of the sham and experimental groups. There was no significant change in total thiol concentrations in the hippocampus and cortex of sham and experimental groups (Figure 4).

Effects of crocin on glutathione peroxidase levels

As shown in Figure 5, there was no significant difference in glutathione peroxidase concentration in the hippocampus and cortex of sham, 6-OHDA-lesioned rats and lesioned groups treated with crocin at 30 and 60 mg/kg at the end of week 6.

Effects of crocin on passive avoidance learning

As shown in Figure 6, the step-through latency of 6-OHDA-lesioned rats was shorter than the sham group rats

at the end of week 6 ($p < 0.05$, Figure 6). Moreover, treatment of lesioned rats with crocin at a dose of 30 mg/kg increased the latency as compared with lesioned rats ($p < 0.05$).

DISCUSSION

In the present study, we evaluated the effect of crocin on biochemical and behavioral parameters using the 6-OHDA rat model, because this model imitates the pathological and biochemical features of PD, such as oxidative stress, mitochondrial dysfunction and apoptosis¹⁹. Oxidative stress, imbalance of free radicals and antioxidants, plays a critical role in the pathogenesis of PD. Dopaminergic neurons are particularly sensitive to oxidative stress because of their low antioxidant capacity, as evidenced by low intracellular glutathione²⁰. A number of studies have also demonstrated that the neurotoxicity of 6-OHDA for the modeling of PD is due to its oxidation, the formation of various oxidants and free radicals and the depletion of reduced glutathione, which leads to lipid peroxidation, protein damage and

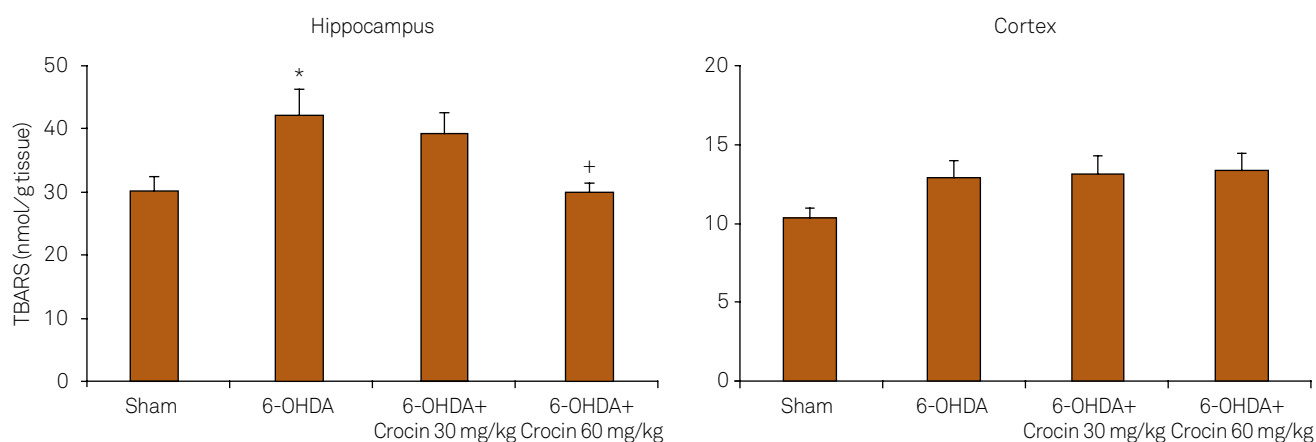


Figure 2. Lipid peroxidation levels (thiobarbituric acid reactive substance [TBARS]) in the hippocampus and cortex of the sham, 6-OHDA-lesioned rats and lesioned rats treated with crocin at doses of 30 and 60 mg/kg at the end of week 6. Data are mean \pm SEM for eight animals in each group. * $p = 0.01$ vs sham group, + $p < 0.05$ vs 6-OHDA-lesioned group.

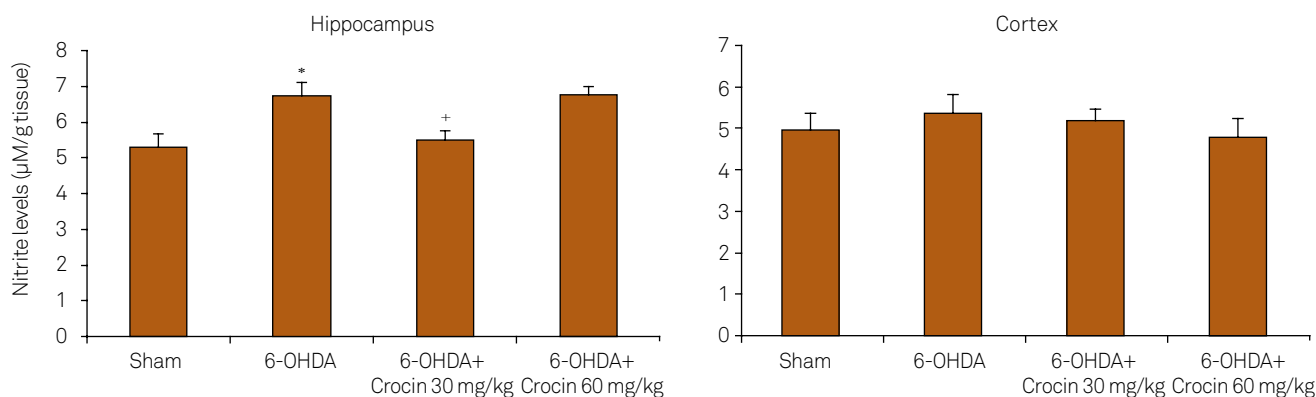


Figure 3. Nitrite levels in the hippocampus and cortex of the sham, 6-OHDA-lesioned rats and lesioned rats treated with crocin at doses of 30 and 60 mg/kg at the end of week 6. Data are mean \pm SEM for eight animals in each group. * $p < 0.05$ vs sham group, + $p < 0.05$ vs 6-OHDA-lesioned group.

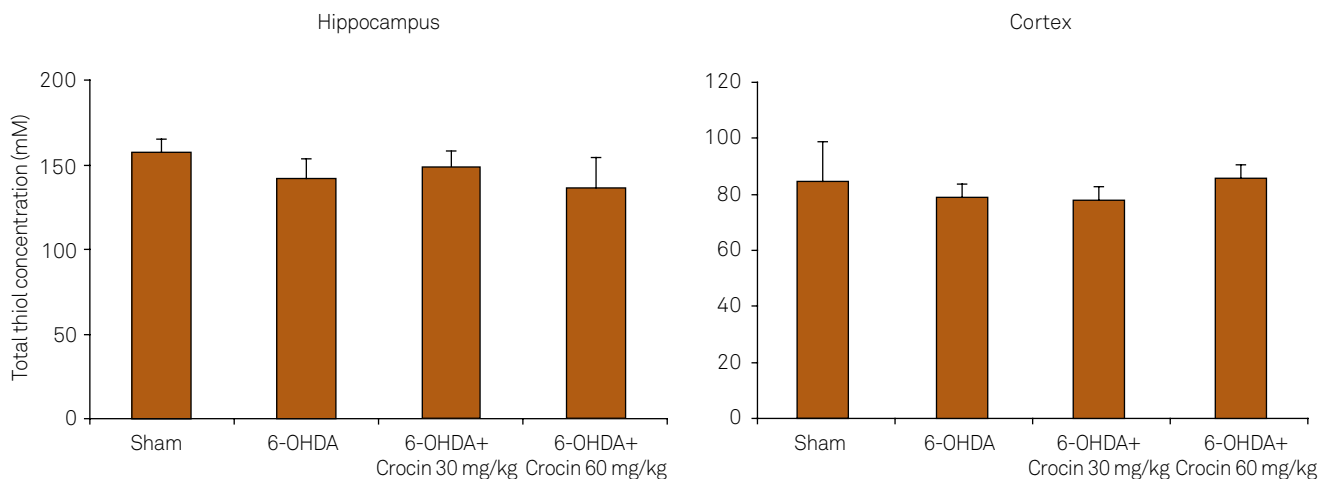


Figure 4. Total thiol concentrations in the hippocampus and cortex of the sham, 6-OHDA-lesioned rats and lesioned rats treated with crocin at doses of 30 and 60 mg/kg at the end of week 6. Data are mean \pm SEM for eight animals in each group.

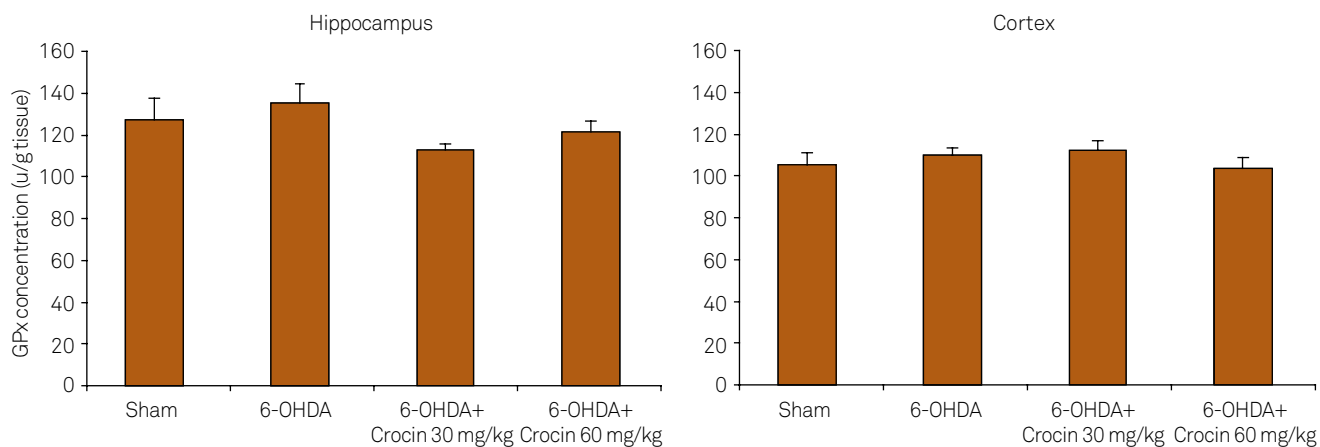


Figure 5. Glutathione peroxidase concentrations in the hippocampus and cortex of the sham, 6-OHDA-lesioned rats and lesioned rats treated with crocin at doses of 30 and 60 mg/kg at the end of week 6. Data are mean \pm SEM for eight animals in each group.

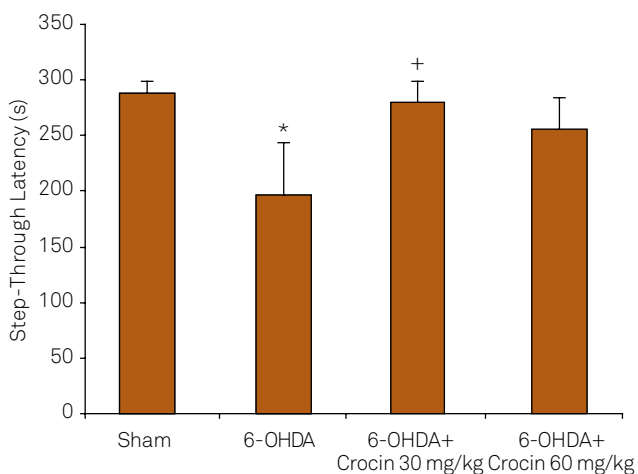


Figure 6. Step-through latencies in the passive avoidance test in the sham, 6-OHDA-lesioned rats and lesioned rats treated with crocin at doses of 30 and 60 mg/kg at the end of week 6. Data are mean \pm SEM for eight animals in each group. * p < 0.05 vs sham group, + p < 0.05 vs 6-OHDA-lesioned group.

ultimately degeneration of the neurons⁹. In the present study, we observed an elevated levels of TBARS in the hippocampus of PD rat brains, which was not accompanied by a depleted glutathione peroxidase level. This was, in part, in agreement with previous observations²¹. Our findings also revealed that long-term treatment with crocin reduced the TBARS levels in the hippocampus of parkinsonian rats. This is in agreement with previous studies which that report the antioxidant effects of crocin in other models. For example, Rajaei et al.¹³ reported that crocin attenuated hepatorenal oxidative damage in streptozotocin-induced diabetic rats, as indicated by a significant decrease in TBARS levels and an elevation in total thiol concentrations. Furthermore, the radical-scavenging and neuroprotective effects of crocins have also been shown in several *in vitro* models^{12,16}. For instance, Zhang et al.¹⁶ have recently shown that crocin protects PC12 cells against 1-methyl-4-phenylpyridinium-induced injury. Collectively, it seems that the antioxidant activity of crocin in the hippocampus in a 6-OHDA model of PD is related to its antioxidant and radical scavenging activity.

A large body of evidence also supports the involvement of inflammation in the pathogenesis of PD. Microglial activation is considered as a rapid cellular response to inflammation. Activation of microglia induces cytotoxic mediators such as NO and inflammatory cytokines, which may contribute to the PD progression²². NO production resulting from induced NOS gene expression and subsequent iNOS enzyme activation is a primary contributor to the inflammatory response^{22,23}. Examination of postmortem PD brains has revealed robust microgliosis and the presence of high levels of iNOS expression in the substantia nigra (SN) compared with control brains²⁴. It has also been reported that unilateral injection of 6-OHDA into the SNc increased NOS expression in the SN and striatum of PD rats²⁵. Along with this, the present findings showed that the 6-OHDA increased the levels of the nitrite, stable NO metabolite, in the hippocampus of parkinsonian rats. The results also showed that treatment with crocin attenuated the nitrite levels in this tissue. To our the best of knowledge, this is the first study reporting the anti-inflammatory effects of crocin through reducing NO levels in a 6-OHDA model of Parkinson's disease, which is in agreement with its anti-inflammatory effects that have previously been reported¹⁴. In this context, it has been reported that crocin inhibited the lipopolysaccharide-induced NO release from cultured rat brain microglial cells¹⁴. Moreover, treatment with crocin decreased NO levels and NOS activity in cortical microvascular homogenates in an ischemic model in rat²⁶.

In the present study, 6-OHDA injections also produced memory deficit, which acts by increasing oxidative stress within the brain of rats. Previous studies have also demonstrated that 6-OHDA could produce cognitive impairments in animals, and oxidative stress has been shown to play an important role in memory impairment²⁷. Reactive oxygen species induced by 6-OHDA can react with biological target molecules and contribute to increased neuronal damage and death through protein oxidation, DNA damage, and peroxidation of membrane lipids. In our study, the passive avoidance test was used to examine whether crocin could improve memory of parkinsonian rats. This task is based on the motivation of passive avoidance from the fear of foot shock. Crocin at a dose of

30 mg/kg increased the step-through latency during the testing session as when compared to parkinsonian rats, in the other words, it produced the amelioration of retention memory in parkinsonian rats. Consistent with this, memory-enhancing effects of crocin have previously been reported in other models, such as cerebral ischemia.

However, it looks like that 60 mg/kg crocin is more efficient in decreasing TBARS in the hippocampus compared to the 30 mg/kg dose, but 60 mg/kg crocin did not significantly affect the behavior impairment. This discrepancy could be related to the fact that several other factors, rather other than reactive oxygen species and oxidative stress, are also involved in the development of cognitive impairments in PD. These factors include the imbalance in NO production and increased nitrosative stress²⁸, dysfunction of the cholinergic system²⁹, neuroinflammation and apoptosis³⁰. For instance, Kuhad and Chopra²⁸ have reported a significant increase in nitrite levels in the cortex and hippocampus of diabetic rats having with cognitive deficits. In our study, nitrite levels were also significantly increased in the hippocampus of parkinsonian rats. Excessive production of NO by increased iNOS expression leads to the formation of an extremely potent oxidizing agent, peroxynitrite (ONOO⁻), which causes neuronal death¹⁰. Peroxynitrite, which is formed by reaction between superoxide and NO, reacts with many biological target molecules and damages the neurons by oxidizing or nitrating proteins, lipids, and DNA¹¹. In fact, the cell membrane has no significant barrier against diffusion of peroxynitrite into cells, thus allowing peroxynitrite to induce DNA damage and to mediate the activation of apoptosis pathways¹⁰. Taken together, the data presented here suggest that the improvement of memory by 30 mg/kg crocin might be mediated, at least in part, by decreased production of NO and inhibition of nitrosative stress in the hippocampus.

In conclusion, the present study demonstrated that the crocin acts as an antioxidant and anti-inflammatory agent in the hippocampus of parkinsonian rats and could improve aversive memory, at least in part, through reducing nitrite levels in the brain. Considering the safety of crocin in clinical trials, it might be used as an adjunct therapy for improvement of memory impairments in PD in the future.

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