



Neuroprotective effect of crocin on substantia nigra in MPTP-induced Parkinson's disease model of mice

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Abstract

Parkinson's disease is caused by damage to substantia nigra dopaminergic neurons. Factors such as oxidative stress, inflammatory factors, and acetylcholinesterase activity may induce this disease. On the other hand, crocin—one of the active ingredients of saffron—has anti-oxidant and anti-inflammatory properties. This study was performed to evaluate the protective effect of crocin to decrease dopaminergic neuron damage and Parkinson's disease complications induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). A set of 24 male BALB/c Mice were divided randomly into four groups: (1) MPTP group receiving 30 mg/kg MPTP for 5 days; (2) MPTP + crocin group receiving 30 mg/kg MPTP for 5 days and 30 mg/kg crocin for 15 days; (3) NS group receiving normal saline for 5 days; and (4) NSIG group receiving normal saline intraperitoneally for 5 days and also normal saline by gavage for 15 days. After the treatment period, pole and hanging motor tests were performed in all groups. Then, the brains of all the animals were removed and fixed in formalin, prepared according to routine histologic methods and cut into sections of 5 μ m thickness. Prepared sections were stained by immunohistochemistry techniques and toluidine blue to detect tyrosine-hydroxylase (TH)-positive neurons and dark neurons, respectively. Finally, the mean number of these cells were calculated by stereological methods and compared with the statistical tests in different groups. The results showed a significant increase in the time taken for the animal to fall from the pole in the MPTP group in comparison with other groups ($P < 0.001$). The time taken for them to stay on the wire in the hanging test decreased significantly in the MPTP group compared to the other groups ($P < 0.001$), while in the MPTP + crocin group, the time to falling decreased ($P < 0.05$) and the time staying on the wire increased ($P < 0.001$) compared to the MPTP group. The number of TH-positive neurons in the MPTP group also decreased significantly in comparison with saline and MPTP + crocin groups ($P < 0.001$). The number of dark neuron sin the MPTP group increased significantly as compared with saline and the MPTP + Crocin groups ($P < 0.001$). Our results showed that crocin improves MPTP-induced Parkinson's disease complications and decreases cell death in the substantia nigra.

Keywords Crocin · Dark neuron · MPTP · Substantia nigra · Tyrosine hydroxylase

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease in the world, and many people around the world suffer from this disease (Mounsey et al. 2015). The

disorder is associated with symptoms such as tremor, muscle stiffness, imbalance and lag in movements (Xia et al. 2017; Cacabelos 2017). It has been proven that degeneration of dopaminergic neurons in the dense part of substantia nigra (SN) and a reduction in the amount of dopamine in the brain are the cause of this disease (Ahn et al. 2017). SN is a motor nucleus in the midbrain that consists of two parts: dense and reticular. The dense part of SN has many dopaminergic neurons and its role is to control motor activity. Dopaminergic fibers of SN extend to various regions, including striatum, and play an important role in the motor activity of the brain (Ahn et al. 2017; Hodge and Butcher 1980; Haber et al. 2000). Dopaminergic neurons in SN are extremely vulnerable to various factors, including oxidative stress,

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and damage to these neurons leads to a decrease in dopamine neurotransmitters and to Parkinson's symptoms (Singh et al. 2017). Toxins are used to induce animal models of PD; one of the most commonly used is 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Al-Jarrah and Erekat 2017; Hosseinzadeh et al. 2017; Yan et al. 2018; Zhang et al. 2018). MPTP is a selective neurotoxin that is used to induce PD in animal models (Hami et al. 2015; Fisher et al. 2018; Qureshi and Paudel 2011; Meng et al. 2017). This substance selectively destroys dopaminergic neurons in the SN and consequently leads to the exhibition of Parkinson's symptoms (L'Episcopo et al. 2010). MPTP substance is not toxic itself, but it can pass through the blood–brain barrier (Chao et al. 2009) and be metabolized by the monoamine oxidase B (MAO-B) enzyme of glial cells, becoming converted to MPP⁺ (1-methyl-4-phenylpyridinium), which is a neurotoxin. MPTP, by creating various mechanisms such as severe increase of oxidative stress, inflammation, apoptosis, and increase of acetylcholinesterase (AChE) activity, can lead to damage of dopaminergic neurons and the occurrence of Parkinsonism (Hauser and Hastings 2013; Nadjar et al. 2009; Mogi et al. 1999; Emmett and Greenfield 2005; Zhang et al. 2013). So far, several drugs have been introduced to treat PD, but unfortunately none have been beneficial and now the disease has become a major global problem (Ahn et al. 2017). Crocin is one of the active ingredients of saffron and is known as a potent antioxidant. Crocin is water soluble and has a wide range of pharmaceutical activities (Sarshoori et al. 2014; Hosseini et al. 2017). Crocin has neuroprotective (Mohammadzadeh et al. 2018), anticancer, and anti-apoptotic effects (Aung et al. 2007). In their studies, Mohammadi et al. (2018) concluded that crocin has a neuroprotective effect on oxidative stress-induced neural damage. Crocin is also able to play an anti-inflammatory role (Nam et al. 2010). In addition, it has been proven that administration of crocin is also able to prevent the increase in activation of AChE (Dorri et al. 2015). Therefore, according to these properties of crocin, we decided to evaluate the neuroprotective effects of crocin against dopaminergic neuron injury and PD complications in a mouse model of PD induced by MPTP.

Materials and methods

This study was conducted at Mashhad University of Medical Sciences, Mashhad, Iran, and experimental protocols were approved by the Institutional Animal Care and Use Committee.

Materials

The crocin powder used in this study was purchased from Bu Ali Research Institute, Mashhad University of Medical Sciences. MPTP powder (M0896, Sigma), primary antibody, anti-tyrosin hydroxylase (TH) (ab191486) and secondary antibody (ab97051) were purchased from Abcam (Cambridge, UK).

Animals

Twenty-four male BALB/c mice aged 8–10 weeks with an average weight of 40 ± 2 g were obtained from the animal center of Mashhad University of Medical Science, Iran. For 1 week before the start of the experiments, the animals were housed in special cages at room temperature ($20 \pm 2^\circ\text{C}$), suitable moisture and 12 h light/dark cycle in the animal center for adaptation with condition and stress relief. During this time, water and food were provided sufficiently to the animals.

Study design

The 24 male mice were divided randomly into four groups, each with six mice ($n=6$):

- (1) MPTP group: the animals received 30 mg/kg MPTP intraperitoneally for 5 days (Xiao-Feng et al. 2016; Matsui et al. 2009).
- (2) MPTP + crocin group: the animals were received 30 mg/kg MPTP intraperitoneally for 5 days and co-administration of 30 mg/kg crocin by gavage was prescribed half an hour before injection of MPTP. It should be noted that the administration of crocin continued until the completion of motor tests (10 days after the last dose of MPTP).
- (3) NS group: the animals received normal saline intraperitoneally for 5 days.
- (4) NSIG group: the animals were administered normal saline intraperitoneally for 5 days and, after 30 min, normal saline was administered by gavage for 15 days. (This group was considered for comparison with MPTP + crocin group).

For evaluating of MPTP-induced Parkinsonism, 7 and 10 days after the last received of MPTP dose, motor tests were performed to determine changes in muscle strength and motor power of the animals.

Movement tests

Pole test

Mice were placed on the top of a vertical pole (height 55 cm with a diameter 8 mm). The time taken by the mice to fully traverse the length of the pole and reach the bottom, and eventually place four hands and feet on the flat surface below, was then recorded (Meng et al. 2017).

Hanging test

The mice were placed on a wire (length 100 cm, diameter 2 mm and height 40 cm from the ground). The wire was then rotated to place the mice in hanging state. The time for which the mouse could hold itself was recorded, then the average time for each group was compared with that of other groups.

Both tests were repeated three times for each mouse and each time with a 2 min rest period.

Histology study

After completing motor tests, all the mice were sacrificed according to ethical principles. Then, by cutting along the sagittal suture of the skull, the brains were removed carefully and fixed in 10% formalin for 10 days. The samples were then dehydrated by alcohol, cleared by xylene and embedded in Paraffin. Serial sections were then made from Bergama with a thickness of 5 μ m in the coronal section at a distance of 3 mm up to 3.7 mm. From each block, we provided six successive sections, three of which were used for immunohistochemistry slides and three for toluidine blue staining.

Immunohistochemistry

The tissue sections were deparaffinized by xylene, rehydrated by alcohol and washed with phosphate buffered saline (PBS) for 5 min. Then, heat-induced antigen retrieval was performed for 15 min at 96 °C. In the next step, samples were incubated with BSA (100 ml PBS + 0.1 gr BSA + 10 ml Triton X-100) at room temperature for 30 min. To block endogenous peroxidase, the samples were placed in 0.03% H₂O₂ in PBS for 15 min and after washing, incubated with goat serum for 20 min at room temperature, then incubated overnight with the anti-TH primary antibody. After washing with PBS, samples were exposed to secondary antibody (goat anti-rabbit IGg) with 1:100 concentrations for 90 min at room temperature and finally, after washing with PBS, tissue sections were

treated with 3,3'-diaminobenzidine (DAB) for 10 min and counterstained with hematoxylin (Rajabzadeh et al. 2012; Jalayeri-Darbandi et al. 2018).

Toluidine blue

The brain tissue sections were deparaffinized with xylene, rehydrated by alcohol and washed with distilled water. The sections were then stained with toluidine blue for 20 s. After staining, the slides were washed with water and dehydrated in ethanol. Finally, the slides were placed in xylene and mounted (Baghishani 2018; Bagheri-Abassi et al. 2015).

Stereology method

After staining, the slides were observed and photographed using a light microscope (Olympus BX51, Japan) with a $\times 40$ objective lens (UPlan FI, Olympus, Tokyo, Japan). Following transfer to a computer, the number of TH-positive cells and dark neurons were counted on the computer monitor by using rectangular grids placed randomly in the investigated areas. Finally, the mean numbers of TH-positive cells and dark neurons per unit area (N_A) were calculated using the following formula:

$$N_A = \frac{\sum \bar{Q}}{a/f \cdot \sum P}$$

In this formula, " N_A " is the number of neurons in area, " $\sum \bar{Q}$ " is the sum of counted particles in the sections, " a/f " is the area associated with each frame, and " $\sum P$ " is the sum of frame-associated points hitting the defined space (Mohammadipour et al. 2014).

Statistical analysis

Statistical analysis was performed using the SPSS 16 software for Windows, and the data obtained from cell counting methods were reported as mean \pm SEM. The data were then analyzed using one-way analysis of variance (ANOVA) and Tukey statistical tests. $P < 0.05$ was considered statistically significant.

Results

Evaluation of motor tests

Pole test

The results of this test showed that there was no significant difference between the mean time taken for the animal to come down from the pole on day 17 in the MPTP and

MPTP + crocin groups in comparison with saline groups. Also, there was no significant difference between the mean time taken for the animals to come down from the pole in the MPTP + crocin group as compared to the MPTP group (Fig. 1a).

The results of the pole tests at day 10 showed a significant increase in mean time taken for the animal to come down from the pole in the MPTP group in comparison with saline groups ($P < 0.001$). The mean time taken to come down in the MPTP + crocin group was significantly lower than in the MPTP group ($P < 0.05$). Also, the MPTP + crocin group exhibited a significant increase as compared to the NS ($P < 0.05$) and NSIG groups ($P < 0.05$) (Fig. 1b).

Hanging test

Evaluation of the results of the hanging test on day 17 showed that there was a significant decrease between the mean time spent by the animal in hanging in the MPTP group compared with the NS ($P < 0.01$) and NSIG ($P < 0.001$) groups, while there was no significant difference in the mean time taken for the animal to hang in the MPTP + crocin group as compared to the MPTP and saline groups (Fig. 2a).

The results of this test on day 10 showed that there was a significant decrease between the mean time spent by the animal hanging in the MPTP group as compared to saline groups ($P < 0.001$). There was a significant increase in

the time in the MPTP + crocin group as compared to the MPTP group ($P < 0.001$). The results revealed that the MPTP + crocin group had a significant decrease in comparison with saline groups ($P < 0.001$) (Fig. 2b).

Histological study

Immunohistochemistry

Evaluation of samples prepared for the immunohistochemistry techniques showed that TH-positive neurons of the dense part of SN were visible in different groups, although these neurons were less visible in the MPTP and MPTP + crocin groups than saline groups (Fig. 3). The results of neuron counting in immunohistochemistry staining showed that TH-positive cells in MPTP groups ($P < 0.001$) and MPTP + crocin ($P < 0.01$) exhibited a significant decrease in comparison with the saline group. While the number of TH-positive cells in the MPTP + crocin group showed a significant increase as compared to the MPTP group ($P < 0.001$) (Fig. 4).

Toluidine blue

Examining the slides prepared with toluidine blue staining showed that dark neurons in the dense part of SN are visible in different groups. These neurons were much more evident in the MPTP group, while MPTP + crocin groups

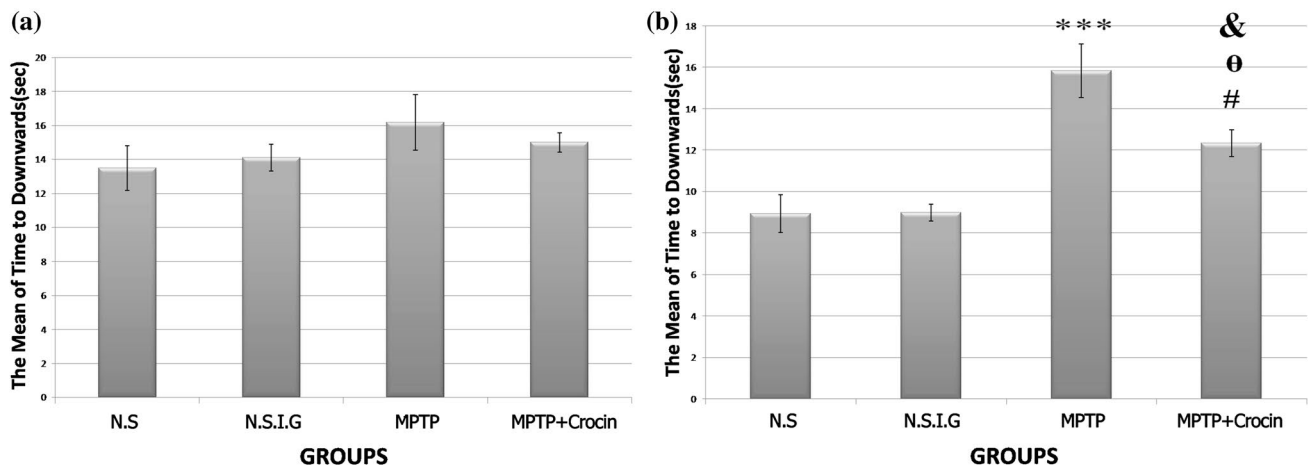


Fig. 1 **a** Average time taken for mice to come down from the rod in the pole test on day 17 after the last intervention in different groups. There was no significant difference in the mean time taken to come down from the rod in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) group as compared with the saline groups. There was no significant difference in the mean time taken to come down in the MPTP + crocin group as compared to the MPTP group. There was also no significant difference in the time in the MPTP + crocin group as compared to the saline groups. **b** Comparison of mean time taken for mice to come down from the rod in the pole test on

day 10 after the last intervention in different groups. There was a significant increase in the mean time taken to come down from the rod in the MPTP group as compared with the saline groups ($***P < 0.001$), while there was a significant decrease in the mean time taken to come down in the MPTP + crocin group as compared to the MPTP group ($^{\#}P = 0.39$). Also, the MPTP + crocin group had a significant increase as compared to normal saline (NS) ($^{\theta}P < 0.05$) and normal saline intraperitoneally and by gavage (NSIG) ($^{\&}P < 0.05$) groups

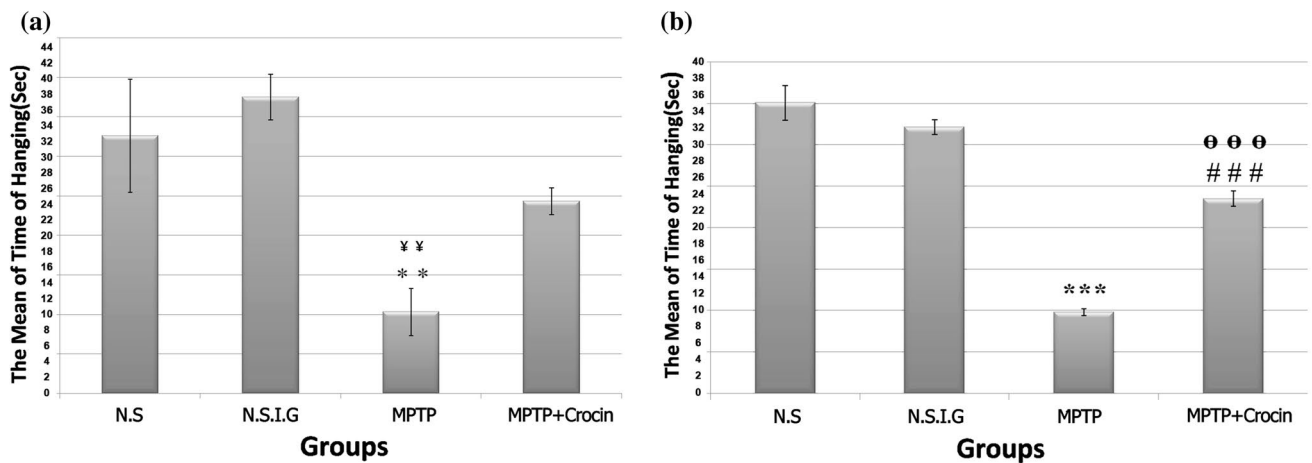


Fig. 2 a Comparison of the average time spent by the mice in hanging in the Hanging test on day 17 after the last intervention in different groups. There was a significant decrease in the mean time spent hanging in the MPTP group as compared with the NS (** $P < 0.01$) and NSIG (^{YY} $P < 0.001$) groups. There was no significant difference between the mean time in the MPTP+crocine group as compared with the MPTP group. Also, there was no significant difference in the mean time in MPTP+ crocin group as compared to the saline groups.

b Comparison of the mean time spent by mice hanging in the Hanging test on day 10 after the last intervention in different groups. There was a significant decrease in the mean time taken spent hanging in the MPTP group as compared with the saline groups (** $P < 0.001$), while there was a significant increase in the time in MPTP+crocine group as compared to the MPTP group (^{###} $P < 0.001$). The results also showed that the MPTP+ crocin group had a significant decrease in comparison with saline groups (^{ooo} $P < 0.001$)

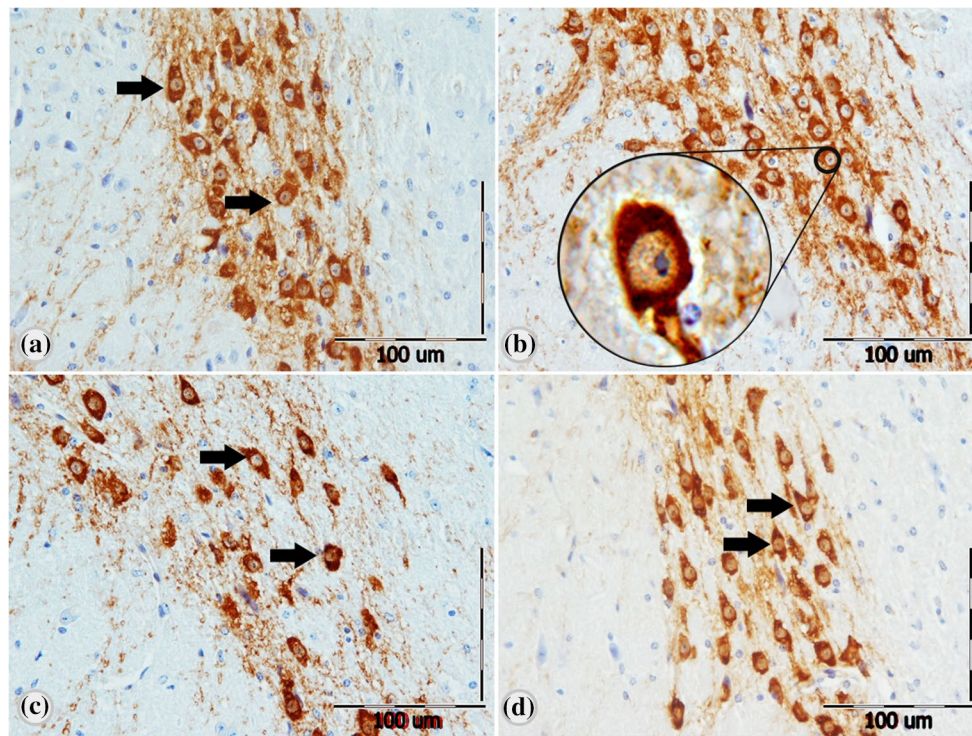


Fig. 3 Tissue sections of the dense part of substantia nigra (SN) implanted in the vicinity of anti-enzyme tyrosine hydroxylase (TH) antibody in different groups. Dopaminergic neurons in the saline

groups are more visible compared to the MPTP+crocine and MPTP groups. **a** NS, **b** NSIG, **c** MPTP, **d** MPTP+crocine. Bar 200 μm, arrows TH-positive cells

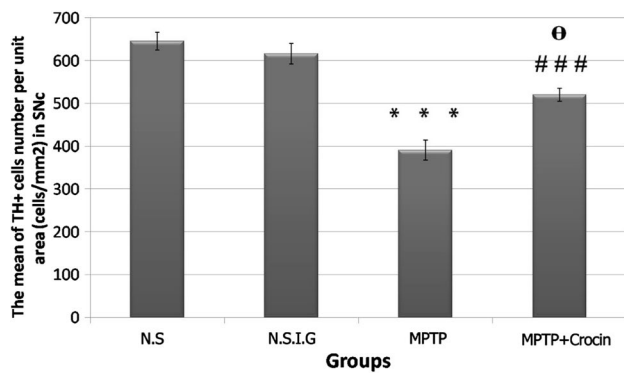


Fig. 4 The mean number of TH-positive neurons in the SN pars compacta in MPTP group showed a significant decrease as compared to that in saline groups (***) $P < 0.001$, while the number of TH-positive neurons in the MPTP+crocine group increased significantly in comparison to the MPTP group (###) $P < 0.001$. The number of these neurons in the MPTP+Crocine group was also significantly lower than in the saline groups (°) $P < 0.05$. SNc Substantia nigra pars compacta

and saline groups had fewer. Cell counts of toluidine blue stained cells showed that the mean number of dark neurons in the MPTP group increased significantly in comparison with saline groups ($P < 0.001$), while the number of dark neurons in the MPTP+crocine group decreased significantly

comparing with the MPTP group ($P < 0.001$). The number of dark neurons in the MPTP+crocine group increased significantly as compared to the NS ($P < 0.001$) and NSIG ($P < 0.001$) groups (Figs. 5 and 6).

Discussion

Our results showed that the number of dopaminergic neurons in the SN pars compacta in the MPTP group was intensively reduced as compared to normal saline groups, but with the administration of crocin plus MPTP, the number of TH⁺ neurons increased significantly in comparison with the MPTP group. Previous studies have also confirmed that MPTP reduces dopaminergic neurons in the SN and leads to the occurrence of PD in mice (Yan et al. 2018; Tunje et al. 2016; Pasquarelli et al. 2017). In the present study, induction of Parkinsonism was performed using MPTP. After injection, MPTP quickly passes through the blood–brain barrier, going to astrocytes, where it is converted to MPP⁺ (active metabolite of MPTP) by the enzyme MAO-B (Sai et al. 2013). This metabolite is also absorbed into neurons via a dopamine transporter (DAT) and is stored in vesicles. Through mechanisms similar to PD, it ultimately leads to a decrease in dopamine levels in neo-striatum and to motor disorders. PD is a progressive

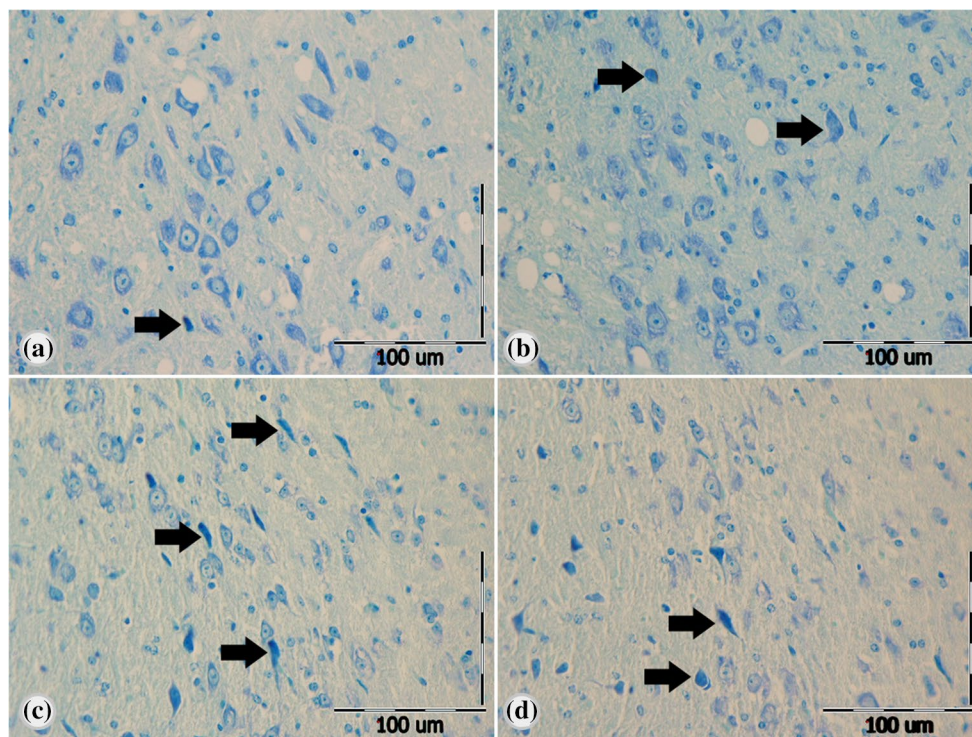


Fig. 5 Tissue sections of the dense part of SN that has been stained by toluidine blue in different groups. Photographs show that more dark neurons are visible in the MPTP group in comparison to other groups, while dark neurons in the MPTP+crocine group are more vis-

ible in comparison to saline groups but are less visible compared with MPTP group. **a** NS, **b** NSIG, **c** MPTP, **d** MPTP+crocine. Bar 200 µm, arrows dark neurons

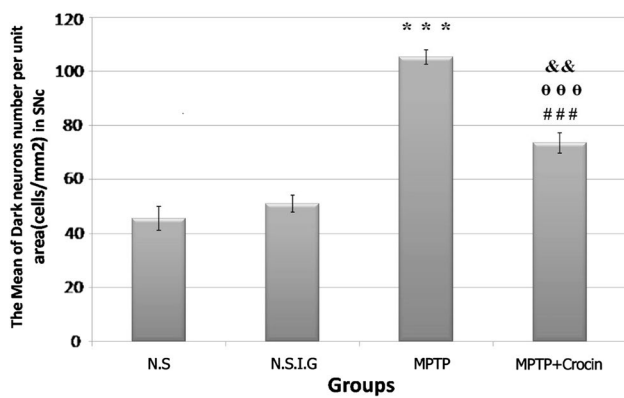


Fig. 6 Comparison of the mean number of dark neurons in the SN pars compacta in different groups. The number of dark neurons in the MPTP group showed a significant increase as compared to saline groups ($***P < 0.001$), while the number of dark neurons in the MPTP+crocine group decreased significantly in comparison with the MPTP group ($###P < 0.001$). The number of these neurons in the MPTP+crocine group showed a significant increase in comparison with the NS ($^{000}P < 0.001$) and NSIG ($^{\&\&}P = 0.001$) groups. SNc Substantia nigra pars compacta

neurodegenerative disease in which many factors are changed, resulting finally, due to excessive damage to dopaminergic neurons, in the appearance of motor symptoms (Martinez and Peplow 2017). One of the important changes is the reduction of neurotrophic factors like BDNF. Neurotrophic factors play a key role in the survival and proper functioning of dopaminergic neurons. Considering that BDNF is expressed in abundance in dopaminergic cells and plays an important role in the survival of these neurons, its reduction can lead to impaired function and also cell destruction (Mogi et al. 1999). Crocin has the ability to prevent the loss of neurotrophic levels of BDNF against neuronal damage, and thus can act as a neuroprotective agent (Dorri et al. 2015). Another major change that occurs in PD is a reduction of acetylcholine and an increase in the amount of AChE (Hilario et al. 2016). Acetylcholine plays a very important role in the functioning of direct and indirect pathways of basal nuclei (Hilario et al. 2016; Lim et al. 2014; Straub et al. 2014). In normal conditions, in the direct pathway active during the voluntary exercise, muscles act smoothly. While in PD, the function of the direct pathway is disturbed, leading to muscle stiffness and slowness of motion. The function of the indirect pathway in normal conditions also prevents the excessive movement at rest, whereas in PD, where this pathway is disturbed, a person experiences tremors (Ahn et al. 2017). On the other hand, it has been proven that crocin has the ability to decrease AChE activity (Dorri et al. 2015). Reducing the activity of this enzyme leads to an increase in acetylcholine. Therefore, another mechanism of crocin shown by this study is the ability to apply a neuroprotective effect by reducing the activity of AChE. The role of oxidative stress is very important in the development of PD because dopaminergic

neurons are highly vulnerable to oxidative stress factors. The electron transport chain in the mitochondria of cells is a huge source of reactive oxygen species (ROS) and, if activated, leads to damage to macromolecules and ultimately to the death of the cell (Hauser and Hastings 2013; Subramaniam and Chesselet 2013). It has also been proven that, in PD, the level of antioxidants decreases intensively. Since many studies have reported the antioxidant properties of crocin (Mohammadi et al. 2018; Hosseini et al. 2016; Najafi et al. 2017), we think that one of the mechanisms by which crocin protects neurons against MPTP in the present study is by controlling the level of oxidative stress. Finally, one result of an increase in oxidative stress and reduction of cellular antioxidants is the activation of apoptotic pathways and death of dopaminergic neurons. Studies have shown that activation of caspase-3 is one of the most important markers of apoptosis and neuronal death. In addition to apoptosis in the neurons, this caspase also causes inflammation in neuroglia and, by increasing factors such as TNF- α and nitric oxide, causes impairment in function and also damage to these cells. Previous studies have also shown that dysfunction of neuroglia and a decrease in the number of these cells is an important factor in the development and progression of PD (Liu et al. 2013). On the other hand, crocin is able to partially prevent the activation of caspase-3 and stop the occurrence of apoptosis. In addition, our results showed that dark neurons in the dense part of SN are visible in different groups. These neurons were much more evident in the MPTP group, while there were fewer dark neurons in MPTP+crocine groups and saline groups. Dark neurons refer to a type of cell death that is neither apoptosis nor necrosis and formation of these cells has been reported in ischemia, epilepsy and hypoglycemia (Gallyas et al. 2008). In all these pathological conditions, excitatory neurotransmitters, such as glutamate, that play a key role in the pathological process of brain disorders are increased (Catarzi et al. 2007). Thus, in the present study, dark neuron formation in the SN after MPTP-induced PD may occur due to oxidative stress, inflammation and an increase in glutamate (Jortner 2006). Also, it has been proven that crocin has the ability to reduce again the level of inflammatory factors such as TNF- α and interleukins that have been increased by toxic substances (Elsherbiny et al. 2016). It seems that, in this study, another pathway for crocin for protecting neurons against MPTP is probably inhibition of apoptotic dark neuron formation and inflammatory factors.

Conclusion

It is concluded that crocin has neuroprotective effects against dopaminergic neuron injury and PD in the mice model of PD induced by MPTP, and it may be possible to use crocin as a therapeutic agent for PD in addition to other routine therapies to reduce progression of the disease in affected patients.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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