

Crocin acts as a neuroprotective mediator against methylphenidate-induced neurobehavioral and neurochemical sequelae: Possible role of the CREB-BDNF signaling pathway

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Methylphenidate (MPH) abuse causes adverse neurobehavioral and neurochemical effects. Some herbal components such as crocin have shown neuroprotective properties. The current study evaluates the potential role of the cyclic AMP response element binding protein (CREB)-brain-derived neurotrophic factor (BDNF) signaling pathway in mediating the neuroprotective effects of crocin against MPH-induced neurotoxicity in rats. Seventy adult male rats were randomly divided into seven groups. Group 1 and 2 received 0.7 ml/rat of normal saline and 10 mg/kg of MPH, respectively. Groups 3, 4, 5, and 6 were treated simultaneously with MPH (10 mg/kg) and crocin (10, 20, 40, and 80 mg/kg, respectively) for 21 days. Group 7 was treated with crocin (80 mg/kg) alone for 21 days. The Morris water maze (MWM) and open field test were used to assess cognitive and locomotor activities. Hippocampal neurotoxicity parameters and levels of BDNF and CREB were evaluated. Simultaneous treatment with various doses of crocin reduced the MPH-induced cognition disturbances and hyperlocomotion. In addition, lipid peroxidation increased with MPH treatment and levels of the oxidized forms of glutathione (GSSG), interleukin 1 beta (IL-1 β), tumor necrosis factor alpha (TNF- α), and Bax increased. MPH treatment decreased levels of the reduced form of glutathione (GSH), P-CREB, Bcl-2, and BDNF in the hippocampus. MPH also reduced activity of superoxide dismutase, glutathione peroxidase, and glutathione reductase in the hippocampus. In contrast, crocin attenuated MPH-induced oxidative stress, inflammation, and apoptosis, and increased levels of P-CREB and BDNF. Thus, crocin – likely via stimulation of the P-CREB/BDNF signaling pathway – displayed neuroprotection against MPH-induced neurotoxicity.

Key words: methylphenidate, crocin, neurotoxicity, CREB, BDNF

INTRODUCTION

Abuse of methylphenidate (MPH), a neurostimulating agent, has increased in recent years (Martins et al., 2006). MPH has been used for the management of attention/deficit hyperactivity disorder (ADHD) in children, and is the drug of choice in the treatment

of ADHD (Leonard et al., 2004). The consequences of chronic use of MPH and its biochemical and behavioral effects remain unclear (Morton and Stockton, 2000; Martins et al., 2006). MPH increases the release of dopamine, norepinephrine, and to a lesser extent, serotonin into synaptic terminals (Morton and Stockton, 2000). MPH also causes hyperstimulation of receptors in the

acute phases, and downregulation of its receptor in the chronic phase (Peles et al., 2015). The pharmacological actions of MPH are similar to cocaine, and this similarity causes a high potential for abuse and addiction (Vendruscolo et al., 2008; Peles et al., 2015). Prolonged abuse of MPH can prompt behavioral changes such as anxiety and depression-like behavior, and also result in cognitive (e.g., learning and memory) impairments in rodent experimental models (Leonard et al., 2004). Previous studies have demonstrated that low doses and acute treatment of MPH can activate the glutamate receptor, whereas high doses and chronic treatment of MPH can inhibit the glutamate receptor and this inhibition is responsible for the adverse cognitive effects (Cheng et al., 2014a; 2014b). MPH has different effects on the GABAergic system during chronic and acute treatment; indeed, previous studies have demonstrated that chronic MPH-induced locomotion and repetitive movements were due to a decrease in GABA transmission, whereas the GABAergic system was not significantly altered following acute MPH treatment (Freese et al., 2012; Goitia et al., 2013). Experimental studies have confirmed the potential effects of MPH on neurodegeneration of several brain areas including the hippocampus, which is involved in cognition and anxiety (Andreazza et al., 2007; Jones and Dafny, 2013). In addition, previous studies suggest that MPH has diverse effects on several brain areas such as the hippocampus, amygdalae, nucleus accumbens, and ventral tegmental area (Gray et al., 2007; Kim et al., 2009; Goitia et al., 2013). Previous studies have demonstrated that MPH abuse can lead to the production of apoptotic proteins, such as Bax, caspase-3, 8, and 9, which can result in DNA fragmentation in various brain regions including the hippocampus and amygdala (Martins et al., 2006; Andreazza et al., 2007). MPH and other neuro-stimulant compounds can cause inflammation, oxidative stress, and mitochondrial dysfunction in brain cells; however the putative mechanism underlying these effects remains unknown (Martins et al., 2006). Interestingly, MPH-induced neurotoxicity seems to be more apparent in certain brain areas, particularly the hippocampus (including CA1, CA2, CA3, and DG subregions) and the amygdala (Morton and Stockton, 2000; Motaghinejad et al., 2017a).

In recent years, there has been a remarkable increase in the use of herbal/natural agents with therapeutic potential (Kanazawa et al., 2017). Natural flavonoids and their products are being extensively evaluated as therapeutic compounds in the treatment of neurodegenerative disorders and some neuropsychiatric disorders induced by drug abuse (Kim, 2005; Kumar and Khanum, 2012). Crocin is a carotenoid chemical agent that is detected in the flowers *Crocus sativus* L

and gardenia (Hosseinzadeh and Talebzadeh, 2005; Lee et al., 2005; Yousefsani et al., 2018). Crocin is the constituent that is the main cause of the color of saffron (Lee et al., 2005; Chen et al., 2008; Khalili et al., 2010). Crocin has been shown to act as an antioxidant (Chen et al., 2008; Mard et al., 2016) and a neuroprotective agent (Mehri et al., 2012). The antioxidant function of crocin is linked to the sugar moiety in the crocin molecule, which plays a key role in its chemical reactivity (Chen et al., 2008). In addition, crocin has potential antidepressant properties in mice and in humans (Hosseinzadeh et al., 2009). The biological effects of crocin are mediated through antioxidant, anti-inflammatory, antiapoptotic, and immunomodulatory activities (Hosseinzadeh et al., 2005; 2009; Khalili and Hamzeh, 2010). Previous research demonstrates that treatment with crocin can counteract oxidative stress by lipid peroxidation reduction, and can improve the functioning of antioxidant enzymes such as superoxide dismutase (SOD) and catalase in some neurodegenerative disorders (Hosseinzadeh et al., 2005; El-Beshbishy et al., 2012). Also, one previous study indicated that chronic treatment of quercetin – an agent that is structurally similar to crocin – can inhibit the manic-like and antioxidant effects of MPH treatment (Kanazawa et al., 2017). Additionally, prolonged treatment with crocin decreased the alcohol-induced increase in levels of interleukin 1 beta (IL-1 β) and tumor necrosis factor alpha (TNF- α) (El-Maraghy et al., 2015). Taken together, all of these reported properties may contribute to the therapeutic potential efficacy of crocin in neurodegenerative disorders related to drug abuse; however, the exact mechanism remains unclear (Tamaddonfard and Hamzeh-Gooshchi, 2010; Firouzi et al., 2010; El-Maraghy et al., 2015). Cyclic AMP response element binding protein (CREB) is a chief transcription factor that is responsible for the regulation of genes related to the synaptic activity and survival of neurons, neuroprotection, and neural plasticity, such as the gene that encodes brain-derived neurotrophic factor (BDNF) (Carlezon Jr et al., 2005; Motaghinejad et al., 2017b). BDNF is a significant neurotrophic factor that primarily supports the development and survival of neurons. BDNF is highly expressed in certain brain regions that are implicated in the control of cognition, emotions, and rewards (Hattiangady et al., 2005; Lee et al., 2005). It has been proposed that crocin may protect hippocampal and frontal neurons from stress-induced damage via an up-regulation of CREB and BDNF; however, this hypothesis has not been confirmed. Therefore, the aim of this study was to evaluate the potential neuroprotective role of crocin against MPH-induced hippocampal impairment, and assess the potential mediating role of the P-CREB-BDNF signaling pathway in this process.

METHODS

Animals

Seventy adult Wistar male rats, weighing between 250–300 g, were purchased from the lab house of the Iran University of Medical Sciences. Rats were kept under controlled condition, i.e., in room temperature ($22\pm0.5^{\circ}\text{C}$) with 12-h light/dark cycles and free access to food and water.

Ethics statement

Our experimental procedure was approved by the Ethical Committee of the Iran University of Medical Sciences (IUMS) and is in accordance with the Guidelines of Animal Ethics and Welfare. The guidelines of the IUMS were constructed in agreement with ARRIVE (Animal Research: Reporting of In Vivo Experiments) procedures (Kilkenny et al., 2010).

Drug

Crocin and MPH were obtained from Sigma-Aldrich (USA) and dissolved freshly in normal saline before administration.

Experimental design

Animals

- Group 1 (i.e., the control group) was administered 0.7 ml/rat, I.P., normal saline for 21 days.
- Group 2 (i.e., MPH) was given MPH (10 mg/kg, I.P.) for 21 days.
- Groups 3, 4, 5 and 6 concurrently received MPH (10 mg/kg, I.P.) and crocin (with doses of 10, 20, 40, or 80 mg/kg, I.P.) for 21 days.
- Rats in Group 7 received crocin (80 mg/kg, I.P.) dissolved in normal saline for 21 days.

It should be noted that, in the groups treated with MPH in combination with crocin, administration of crocin was performed first. MPH was administered after one hour.

During the 17th and 21st days, the Morris water maze (MWM) task, a standard behavioral process for the evaluation of learning and spatial memory, was performed. In day 22, the open field test (OFT) was used as a standard test for the evaluation of locomotor changes in experimental animals. After the 22nd day, all animals were sacrificed and parameters for

oxidative stress, inflammation, and apoptosis were measured in hippocampal tissues. Considering the importance of CREB signaling and its product, BDNF, the influence of crocin on MPH-induced disorders in the CREB signaling pathway was studied in hippocampal tissues (Carlezon Jr et al., 2005; Motaghinejad et al., 2016b; 2017d).

Behavioral method

Morris water maze task (MWM)

The MWM apparatus includes a circular tank (160 cm in diameter and 90 cm in height) that is black in color and filled with water. The apparatus was fixed in the center of the experimental lab. The apparatus was divided into four quadrants (north, east, west, and south), and water was filled to the height of 50 cm. The experimenter remained in the northeast corner of the room during testing. A disk on the platform with 15 cm diameter, which was hidden, was located 1 cm beneath the surface of the water. During the first four days of the experiment, the aforementioned platform was randomly inserted in the same quadrant of the tank. The position of the experimental animal in the tank was recorded by an automated infrared tracking system (CCTV B/W camera, SBC-300 (P), Samsung Electronics Co, Ltd, Korea). The camera was placed 2.4 m above the surface of the water (D'Hooze and De Deyn, 2001; Vorhees and Williams, 2006).

Handling

On the first day, prior to the beginning of the experiment, all rats were positioned one-by-one in a tank that was filled with 40°C water, room temperature ($25\pm2^{\circ}\text{C}$). The experimenter directed the rat to swim and to reach to the quadrant of the tank where the platform was placed. In our experiment, the platform was situated in the southeast quarter of the tank (Vorhees and Williams, 2006; D'Hooze and De Deyn, 2001).

Training procedure

Some discriminating signs (for example, a distinguished picture, window, door) were placed in the room to act as spatial cues for animals, to learn about the location of the platform. As mentioned above, the platform was positioned in the southeast quarter of the MWM tank, at a distance of 25 cm distance from the edge of the tank and 1 cm beneath the surface

of the water. For the evaluation of the learning procedure, each rat was tested using four trials per day, for a total of four days. Each animal was randomly placed in one of the four quarters (north, east, west, and south). During the learning procedure, if the rats found the platform within 60 sec, the trial was automatically closed by a computer. However, if the animals could not reach and found the platform within 60 sec, the experiment was stopped automatically by the computer. In the learning experiment, two parameters were evaluated:

- The time of escape latency, characterized by the time to find the hidden platform.
- Distance traveled, which was quantified as the distance each animal traveled to reach and find the hidden platform.

In the memory assessment procedure, on the fifth day (i.e., probe day), the platform was detached and animals were randomly placed of the water from one of the above-mentioned directions (almost east), and the percentage of the presence of animal in the target quarter (i.e., the southeast quarter) was documented and calculated (D'Hooze and De Deyn, 2001; Vorhees and Williams, 2006).

Open field test (OFT)

The OFT, which is a standard test for assessment of locomotor activity in rodents, was performed according to previous studies (Gould et al., 2009). To assess locomotor activity in each animal, the following four behaviors were assessed:

- Ambulation distance: the distance the rat crossed according to the grid lines.
- Center square entries: the number of times the rat crossed of one of the central red lines with all four paws, and entered into the central square.
- Center square duration: the time the rat spent in the central square.
- Rearing: the number of times the rat stood on their hind legs in the maze (Gould et al., 2009).

Mitochondrial preparations

Animals were anesthetized using I.P. injections of sodium thiopental (50 mg/kg) and the hippocampus was isolated. After homogenization in cold buffer (25 mM 4-morpho linepropanesulfonic acid, 400 mM sucrose, 4 mM magnesium chloride [MgCl_2], 0.05 mM ethylene glycol tetraacetic acid [EGTA], pH 7.3), the homogenized tissues were centrifuged at $450 \times g$ for 10 min. The obtained supernatants were centrifuged again at $12000 \times g$ for 10 min. At the end, the sediments were re-suspended in homogenization buffer and

stored at 0°C . Total mitochondrial proteins in tissues were measured using a Dc protein assay kit (Bio-Rad), (California, USA). In brief, Bradford reagent (one part Bradford: four parts dH_2O) was added to serial dilution series (0.1–1.0 mg/ml) of a known protein sample concentration; e.g., bovine serum albumin (BSA), dissolved in homogenization buffer. These sequential dilution series were prepared and used to create a standard curve. Alternatively 10, 15, 20, 25, and 30 μL of the protein extract (homogenized cell solutions) were added to multiple wells. Bradford reagent was also added to each well. The color density of all wells was read by the plate reader at 630 nm. Finally, protein quantity in the extracts was attained by using the standard curve. These homogenized cell solutions, containing mitochondria of hippocampal cells, were subsequently investigated for the extent of oxidative stress and inflammatory markers (Wieckowski et al., 2009; Motaghinejad et al., 2017d).

Measurement of oxidative stress parameters

Evaluation of lipid peroxidation

To evaluate lipid peroxidation, we assessed malondialdehyde (MDA), a natural by-product. Concisely, 100 μL of SDS lysis solution was added to wells that contained 100 μL of sample solution or MDA standard. After shaking and incubating these wells, 250 μL of thiobarbituric acid (TBA) reagent was added to each well, and wells were subsequently incubated at 95°C for 45–60 min. Following this incubation, the tubes were centrifuged at $1000 \times g$ for 15 min and 300 μL of n-Butanol was added to 300 μL of the supernatant. Then, the tubes were centrifuged for 5 min at $10,000 \times g$. Finally, absorbance was read at 532 nm and the attained results were expressed as nmol/mg of protein (Gheita and Kenawy, 2014; Motaghinejad et al., 2017e).

Evaluation of GSH (glutathione) and GSSG (glutathione disulfide)

To determine levels of GSH (glutathione) and GSSG (glutathione disulfide), 25 μL of the IX glutathione reductase solution and 25 μL of the IX NADPH solution were added to a 96-well plate holding a standard solution of glutathione, or a sample of homogenized solution. Then, 50 μL of IX Chromogen was added to each well and mixed robustly. Finally, absorbance was read at 405 nm for both the GSSG/GSH standard and the sample. Using the standard curve, levels of GSSG/GSH were measured and are given as nmol/mg of protein (Yoo et al., 2007; Gheita and Kenawy, 2014; Motaghinejad et al., 2017e).

Evaluation of manganese superoxide dismutase (MnSOD) activity

SOD function was evaluated using previously described methods (Koracevic et al., 2001; Yoo et al., 2007). SOD activity was calculated using the following equation: $\text{SOD activity} = \{[(A \text{ blank } 1 - A \text{ blank } 3) - (A \text{ sample} - A \text{ blank } 2)] / (A \text{ blank } 1 - A \text{ blank } 3)\} \times 100$ (Koracevic et al., 2001; Yoo et al., 2007).

Evaluation of glutathione peroxidase (GPx) activity

GPx activity was evaluated using previously described methods (Yoo et al., 2007; Gheita and Kenawy, 2014). GPx activity was calculated based on a change in absorbance [$\Delta A_{340}/\text{min}$], using the following equation: $\Delta A_{340}/\text{min} = A_{340\text{nm}} (\text{Start}) - A_{340\text{nm}} (\text{Stop}) / \text{Reaction time (min)}$. Any change in absorbance is directly related to GPx function.

GPx activity: $\Delta A_{340}/\text{min} \times \text{Reaction volume (ml)} \times \text{Dilution factor of the original sample} / \text{The extinction coefficient for NADPH at 340 nm} \times \text{Volumes of the tested sample}$.

Outcomes are given as mU/mg protein (Yoo et al., 2007; Gheita and Kenawy, 2014).

Evaluation of glutathione reductase (GR) activity

GR activity was evaluated using previously described methods (Koracevic et al., 2001, Yoo et al., 2007). GR activity was measured based on a variation in absorbance [$\Delta A_{340}/\text{min}$], using the following equation:

$\Delta A_{340}/\text{min} = A_{340\text{nm}} (\text{Start}) - A_{340\text{nm}} (\text{Stop}) / \text{Reaction time (min)}$, wherein any alteration in absorbance is directly related to GR activity. GR activity: $\Delta A_{340}/\text{min} \times \text{Reaction volume (ml)} \times \text{Dilution factor of the original sample} / \text{Extinction coefficient for NADPH at 340 nm} \times \text{Volumes of the tested sample}$.

Results are given as mU/mg protein (Koracevic et al., 2001, Yoo et al., 2007).

Evaluation of protein expression modifications

Concentrations (i.e., expression of protein) of BDNF, CREB (total and phosphorylated), TNF- α , IL-1 β , Bax, and Bcl-2 in the cell lysates of hippocampal tissue were estimated using a commercially available ELISA kit (Genzyme Diagnostics, Cambridge, USA). Conforming with the guidelines of the ELISA kits, the sensitivity and specificity for the BDNF and CREB (total and phosphorylated) kits were less than 2 pg/ml and 74%. Similarly, sensitivity and specificity for the TNF- α , IL-1 β , Bax, and Bcl-2 kits were less than 5 ng/ml and 76%. In accordance with the guides included in the kits, the procedure was as follows: briefly, wells containing

sheep anti-rat BDNF, CREB (total and phosphorylated), IL-1 β , and TNF- α polyclonal antibodies (Sigma Chemical Co., Poole, and Dorset, UK) were washed three times with washing buffer (0.5 M of sodium chloride [NaCl], 2.5 mM sodium dihydrogen phosphate [NaH_2PO_4], 7.5 mM Na_2HPO_4 , 0.1% Tween 20, pH 7.2). Then, 100 μl of 1% (w/v) ovalbumin (Sigma Chemical Co., Poole, Dorset, UK) solution was added to each well and incubated at 37°C for 1 hr. Following three washes, 100 μl of samples and standards were added to each well and incubated at 48°C for 20 hrs. After three washes, 100 μl of the biotinylated sheep anti-rat IL-1 β or TNF- α antibodies (1: 1000 dilutions in washing buffer containing 1% sheep serum, Sigma Chemical Co., Poole, and Dorset, UK) were added to each well. Next, after 1-hour incubation and three washes, 100 μl avidin-HRP (Dako Ltd, UK) (1: 5000 dilution in wash buffer) was added to each well and the plate was incubated for 15 min. After washing three times, 100 μl of TMB substrate solution (Dako Ltd., UK) was added to each well and then incubated for 10 min at room temperature. Then, 100 μl of 1M H_2SO_4 was added and absorbance was read at 450 nm. Results are given in ng, as amount of IL-1 β /ml or TNF- α /ml. Bax and Bcl-2 in suspension of hippocampal tissues and BDNF and CREB (total and phosphorylated) are reported as pg/ml of suspension in hippocampal tissues (Arican et al., 2005; Demircan et al., 2006; Shi et al., 2010; Lee et al., 2007).

Statistical analysis

The data were analyzed using GraphPad PRISM v.6 Software and averaged in each experimental group and expressed as means $\pm$ standard error of the mean (SEM). Then, the differences between the control and treatment groups were evaluated by analysis of variance (ANOVA). To evaluate the significant of the behaviors, the differences between group means were compared using Tukey's *post test* at a significant level of $p < 0.001$.

RESULTS**Evaluation of escape latency and traveled distance during training days in the MWM**

MPH (10mg/kg) caused a significant increase in escape latency and traveled distance during four days training in the MWM as compared to the control group ($p < 0.001$) (Fig. 1A and B). All doses of crocin inhibited the increase in escape latency and traveled distance following MPH administration, evidenced by a reduction

in escape latency and traveled distance in the crocin groups as compared to MPH (10 mg/kg) treated group ($p<0.001$) (Fig. 1A and B). In addition, the group treated with crocin alone (i.e., crocin in combination with normal saline) did not differ from the control group in escape latency and traveled distance during four days training of MWM (Fig. 1A and B).

Evaluation of swimming speed during training days

There was no difference in swimming speed during training trials across any of the animal groups (Fig. 1C).

Evaluation of percentage of time in the target quarter in the probe trial

10 mg/kg of MPH caused a significant decrease in the percentage of time that the animals spent in the target quarter, in comparison to the control group

($p<0.001$) (Fig. 1D). In all doses used, crocin significantly abolished MPH-induced changes in the amount of time that animals were present in the target quarter (Fig. 1D). In the group treated with crocin alone (i.e., crocin in combination with normal saline) the percentage of time that the animals spent in the target quarter in the MWM did not change significantly when compared to the control group (Fig. 1D).

Effects of various doses of crocin on MPH-induced changes in locomotor activity

As shown in Table I, animals treated with MPH (10 mg/kg) had a lower rate of central square entries, and also spent less time in the central area of the OFT as compared to control group ($p<0.001$). Our study indicated that crocin inhibited this effect of MPH in a dose-dependent manner, and increased the frequency of central square entries and also time spent in the central region of the OFT in the groups treated

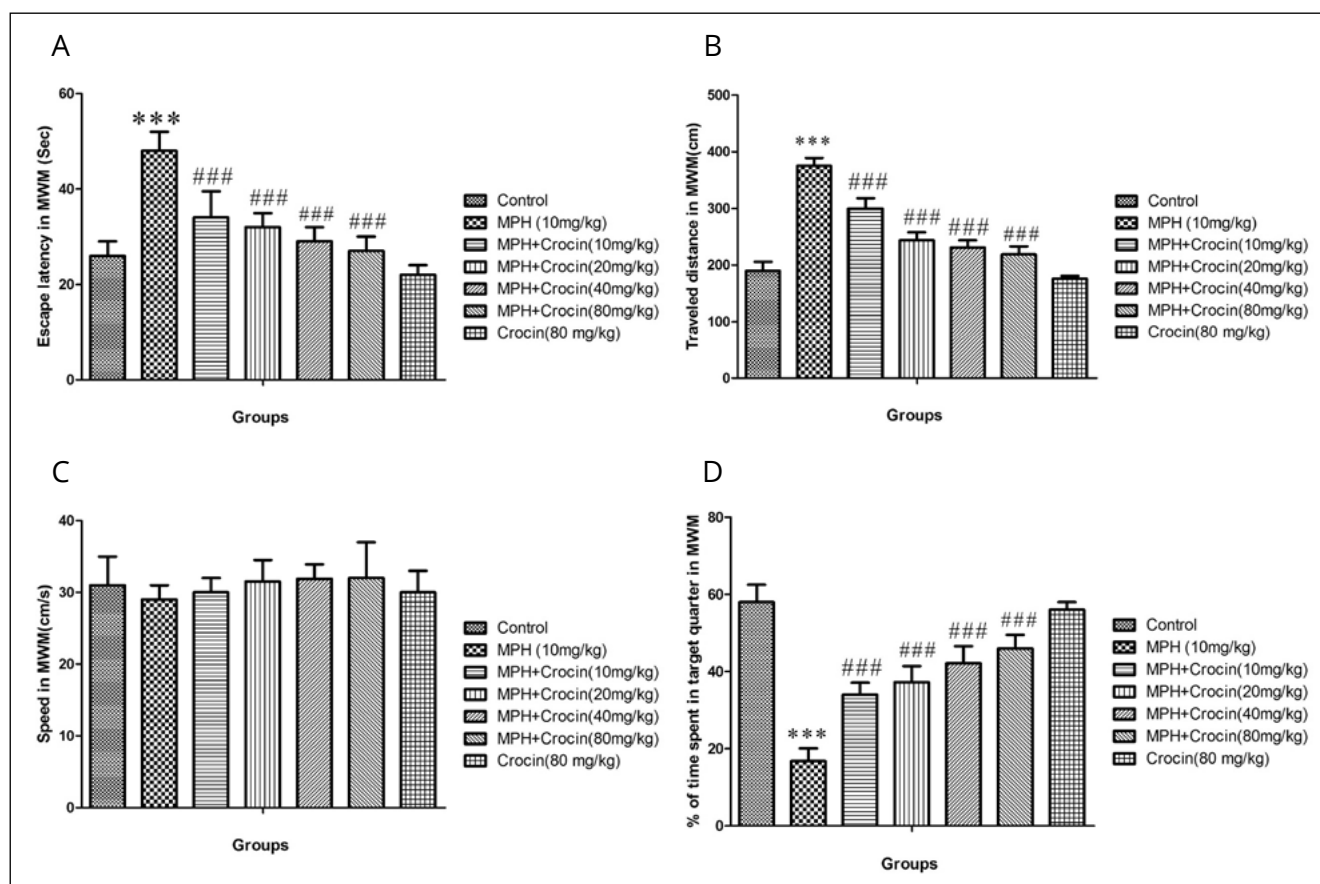


Fig. 1. Mean escape latency (A), distance traveled (B), swimming speed (C) and percentage of time spent in the target quarter during the probe trial (D) in the control group and in the groups treated with 10 mg/kg of MPH and 10 mg/kg of MPH in combination with crocin (10, 20, 40, and 80 mg/kg) across all training days using the Morris water maze (MWM) in rats. All data are expressed as mean $\pm$ SEM ($n=10$). *** $p<0.001$ vs. control. ### $p<0.001$ vs. 10 mg/kg of MPH. MPH: Methylphenidate.

Table I. The effects of various doses of crocin on open field exploratory and locomotor activity on behavior of MPH-treated rats.

Group	Ambulation distance (cm)	Central square entries	Time spent in central square (sec)	Number of rearings
Control	115.0 ± 12.0	29.0 ± 3.0	169.0 ± 9.0	7.0 ± 1.0
MPH (10 mg/kg)	375.0 ± 18.0 ^a	14.0 ± 2.9 ^a	101.0 ± 6.0 ^a	14.0 ± 3.0 ^a
MPH (10 mg/kg) + Crocin (10 mg/kg)	252.0 ± 23.0 ^b	17.0 ± 3.0	109.0 ± 10.0 ^b	12.0 ± 2.0
MPH (10 mg/kg) + Crocin (20 mg/kg)	242.0 ± 12.0 ^b	20.0 ± 3.0 ^b	112.0 ± 12.0 ^b	10.0 ± 2.0
MPH (10 mg/kg) + Crocin (40 mg/kg)	201.0 ± 16.0 ^b	24.0 ± 2.0 ^b	127.0 ± 6.0 ^b	8.0 ± 1.0 ^b
MPH (10 mg/kg) + Crocin (80 mg/kg)	204.0 ± 18.0 ^b	27.0 ± 1.0 ^b	139.0 ± 10.0 ^b	7.0 ± 2.0 ^b
Crocin (80 mg/kg)	120.0 ± 14.0	28.0 ± 1.0	156.0 ± 11.0	7.0 ± 3.0

All data are presented as mean ± SEM, n=10. ^a*p*<0.001 vs. control group. ^b*p*<0.001 vs. MPH group. MPH: Methylphenidate.

with MPH. This difference was statistically significant compared to the group treated with MPH (10 mg/kg) alone (*p*<0.001) (Table I). In addition, in the group treated with crocin alone (i.e., crocin in combination with normal saline), the incidence of central square entries and also time spent in the central area of the

OFT was similar as compared to in the control group (Table I). Animals in the control group, in comparison to the MPH treated group, had a higher frequency of rearing and ambulation distance in the OFT (*p*<0.001). Also, among the animals treated with crocin, there was a significant increase in ambulation distance and

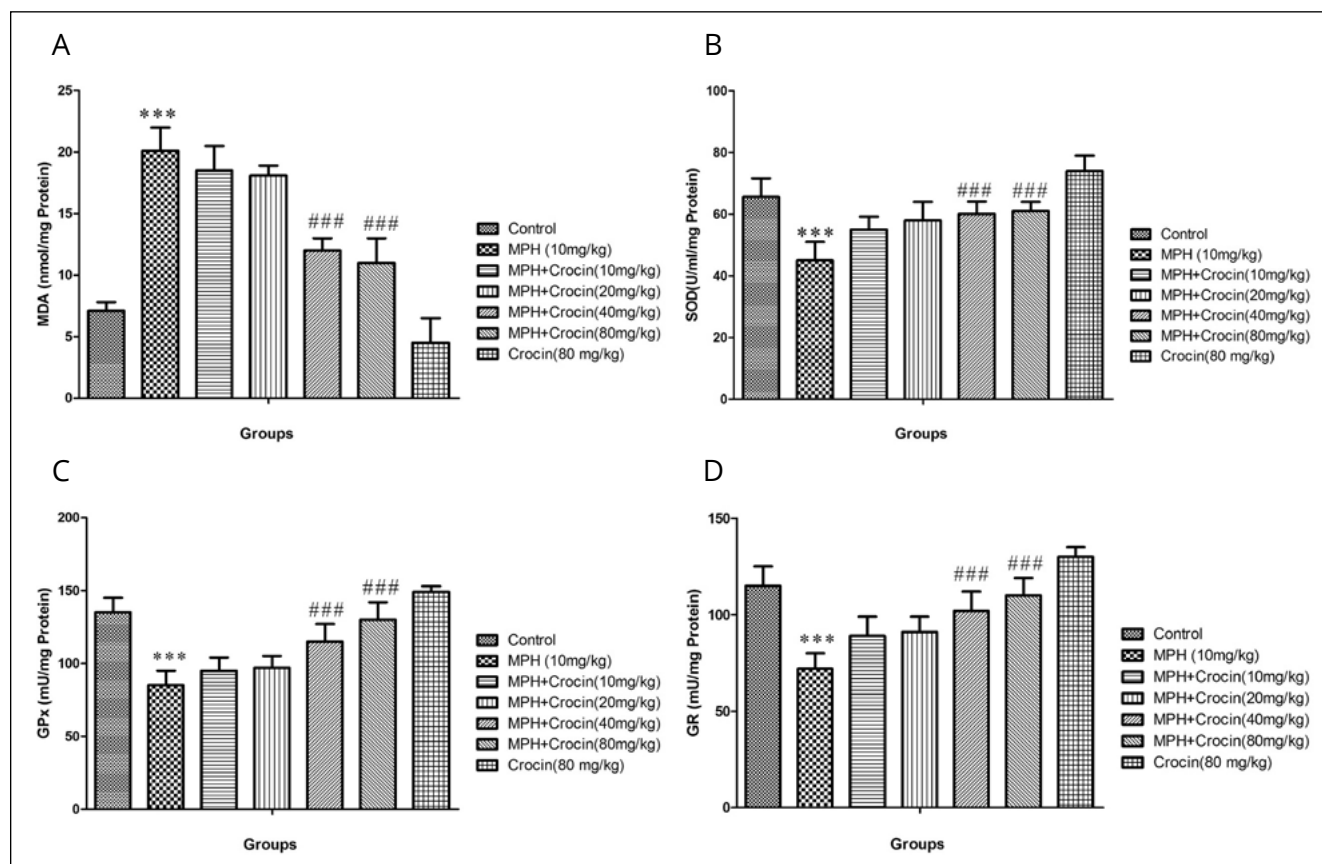


Fig. 2. Effects of various doses of crocin (10, 20, 40, and 80 mg/kg) on MPH-induced lipid peroxidation (A), SOD activity (B), GPx activity (C), and GR activity (D) in rat isolated hippocampus. All data are expressed as mean ± SEM (n=10). *** *p*<0.001 vs. control. ### *p*<0.001 vs. 10 mg/kg of MPH. MPH: Methylphenidate.

Table II. The effects of various doses of crocin treatment on content of mitochondrial GSH and GSSG in MPH-treated rats.

Group	GSH (nmol/mg protein)	GSSG (nmol/mg protein)	GSH/GSSG (nmol/mg protein)
Control group	65.20 ± 5.10	0.88 ± 0.30	73.00
MPH (10 mg/kg)	38.20 ± 5.20 ^a	6.20 ± 0.90 ^a	6.10 ^a
MPH (10 mg/kg) + Crocin (10 mg/kg)	48.60 ± 4.10	4.50 ± 0.05	10.90
MPH (10 mg/kg) + Crocin (20 mg/kg)	52.30 ± 4.00	4.45 ± 0.14	11.50
MPH (10 mg/kg) + Crocin (40 mg/kg)	56.20 ± 5.40 ^b	3.90 ± 0.90 ^b	14.30 ^b
MPH (10 mg/kg) + Crocin (80 mg/kg)	60.30 ± 3.90 ^b	3.20 ± 0.20 ^b	18.75 ^b
Crocin (80 mg/kg)	69.10 ± 8.20	0.82 ± 4.30	80.00

All data are presented as mean ± SEM, n=10. ^a*p*<0.001 vs. control group. ^b*p*<0.001 vs. MPH group. MPH: Methylphenidate.

rearing number in the MPH (10 mg/kg) treated group. This increase significantly differed from the group treated with MPH (10 mg/kg) alone (*p*<0.001) (Table I). Also, in the group treated with crocin alone (i.e., crocin in combination with normal saline), ambulation distance, rearing number, central square entries, and time spent in the central area of the OFT was not significantly different as compared to the control group (Table I).

Effects of various doses of crocin on MPH-induced GSH/GSSG changes

MPH (10 mg/kg) treatment markedly decreased the content of mitochondrial GSH, but increased the levels of GSSG as compared to the control group (*p*<0.001) (Table II). Conversely, high doses of crocin (40 and 80 mg/kg) enhanced GSH content and decreased levels of GSSG levels in MPH-treated animals, in comparison to the group treated with MPH alone (*p*<0.001) (Table II).

Also, in the group treated with crocin alone (i.e., crocin in combination with normal saline), the content of mitochondrial GSH and GSSG did not significantly differ as compared to the control group (Table II).

Effects of various doses of crocin on MPH-induced alterations in oxidative stress parameters

MPH administration significantly increased MDA levels and decreased the activity level of SOD, GPx, and GR in comparison to the control group (*p*<0.001) (Fig. 2A, B, C, and D). Conversely, high doses of crocin (40 and 80 mg/kg) inhibited the MPH-induced increase in MDA levels and decrease in activity of SOD, GPx, and GR (*p*<0.001) (Fig. 2A, B, C, and D). Also, in the animals treated with crocin (80 mg/kg) alone (i.e., crocin in combination with normal saline), there was a decrease in MDA levels, but activity of SOD, GPx, and GR did not significantly differ in comparison with the control group (Fig. 2A, B, C and D).

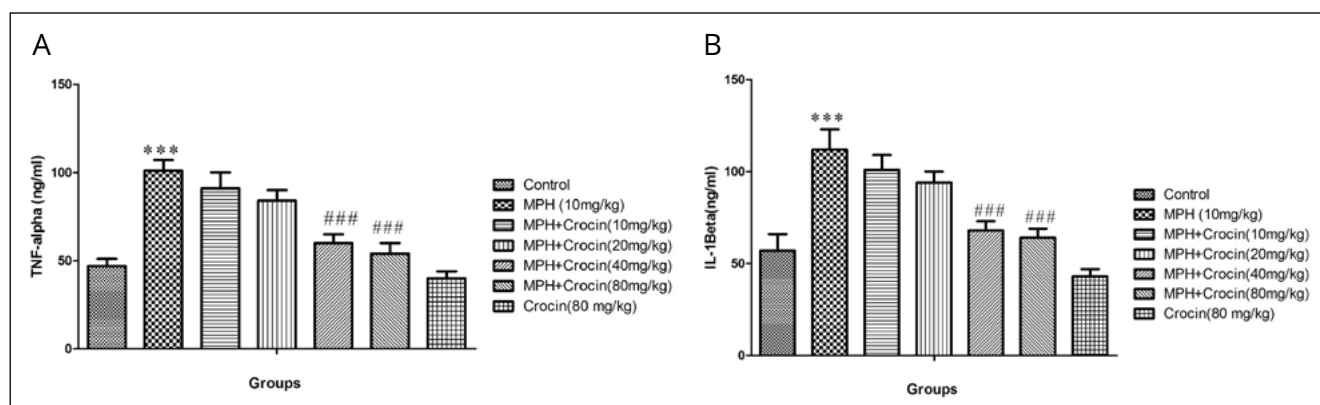


Fig. 3. Effects of various doses of crocin (10, 20, 40, and 80 mg/kg) on MPH-induced alteration in TNF-α (A) and IL-1β (B) levels in rat isolated hippocampus. All data are expressed as mean ± SEM (n=10). *** *p*<0.001 vs. control. ### *p*<0.001 vs. 10 mg/kg of MPH. MPH: Methylphenidate.

Impact of various doses of crocin on MPH-induced increase in inflammatory biomarkers

10 mg/kg MPH caused a significant elevation in levels of IL-1 β and TNF- α , as compared to the control group ($p < 0.001$) (Fig. 3A and B). Conversely, high doses of crocin (40 and 80 mg/kg) prevented the MPH-induced increase in levels of IL-1 β and TNF- α , compared to the group treated with MPH alone ($p < 0.001$) (Fig. 3A and B). Also, in the group treated with crocin (80 mg/kg) alone (i.e., crocin in combination with normal saline), IL-1 β

and TNF- α levels did not significantly differ from the control group (Fig. 3A and B).

Impact of various doses of crocin on MPH-induced alterations in Bax and Bcl-2 levels

MPH (10mg/kg) treatment was associated with increased protein expression of Bax, and reduced protein expression of Bcl-2 in comparison to the control group ($p < 0.001$). On the other hand, crocin (40

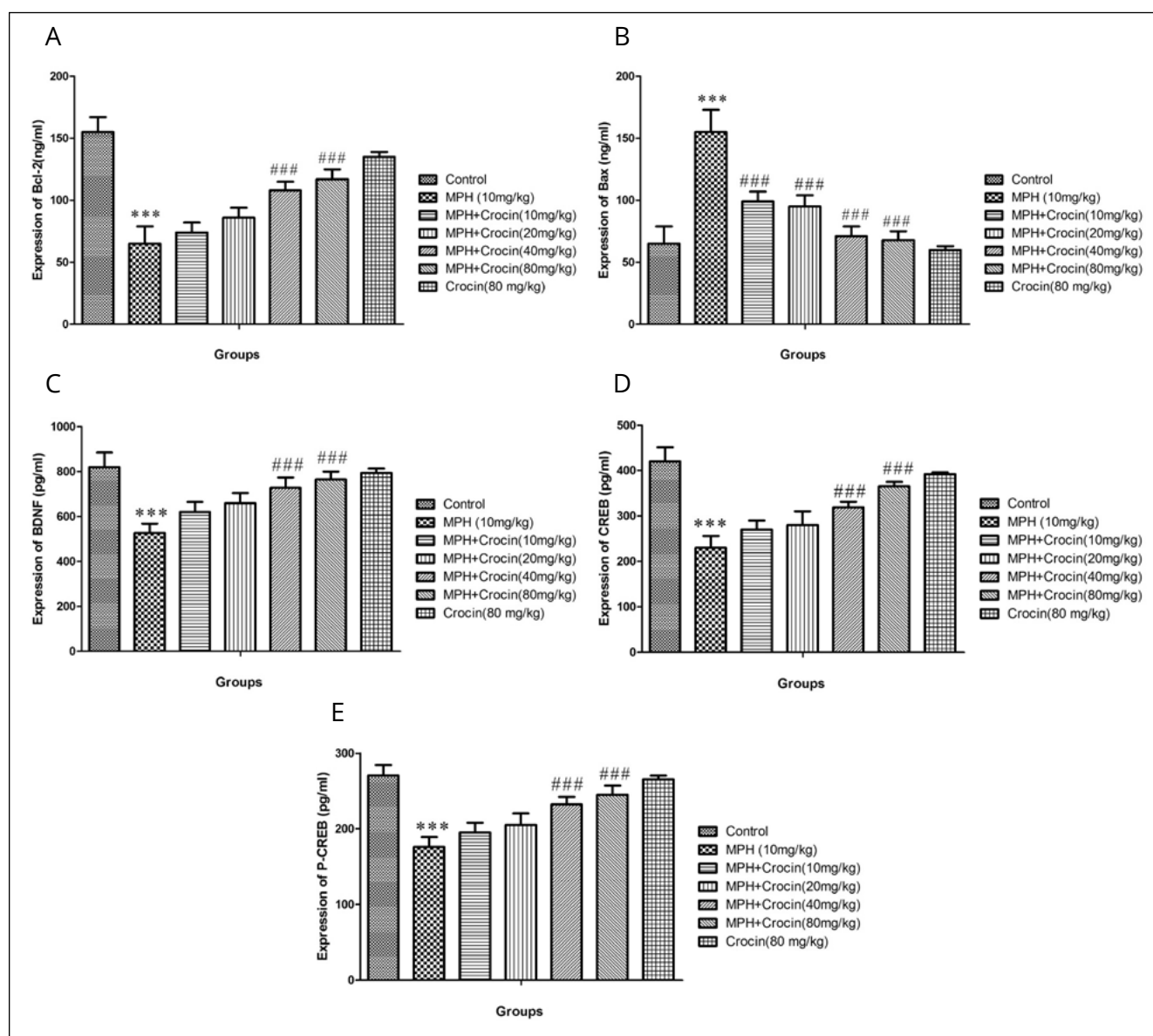


Fig. 4. Effects of various doses of crocin (10, 20, 40 and 80 mg/kg) on MPH-induced alterations in protein expression of Bcl-2 (A), Bax (B), BDNF (C), total CREB (D) and phosphorylated CREB (E) in rat isolated hippocampus. All data are expressed as Mean $\pm$ SEM ($n=10$). *** $p < 0.001$ vs. control. ### $p < 0.001$ vs. 10 mg/kg of MPH. MPH: Methylphenidate.

and 80 mg/kg) enhanced Bcl-2 expression, but – in all examined doses, reduced Bax protein expression in comparison to the MPH treated group ($p < 0.001$) (Fig. 4A and B). Also, in the group treated with crocin (80 mg/kg) alone (i.e., crocin in combination with normal saline), there was no significant difference in levels of Bax or Bcl-2 level in comparison to the control group (Fig. 4A and B).

Impact of various doses of crocin on MPH-induced modification of protein expression in both forms of CREB and BDNF

Relative to the control group, MPH (10mg/kg) treatment significantly decreased the protein expression of both total and phosphorylated forms of BDNF and CREB ($p < 0.001$) (Fig. 4 C, D, and E). Conversely, in MPH-treated animals, administration of high doses of crocin (40 and 80 mg/kg) considerably enhanced protein expression of both total and phosphorylated forms of BDNF and CREB in comparison to the group treated with MPH only ($p < 0.001$) (Fig. 4C, D, and E). Also, in the group treated with crocin (80 mg/kg) alone (i.e., crocin in combination with normal saline), BDNF and CREB (total and phosphorylated) levels were not significant as compared to control group (Fig. 4C, D, and E).

DISCUSSION

The results of the present study demonstrate that several doses of crocin can ameliorate MPH-induced neuro-apoptosis, oxidative stress, and inflammation in the rat hippocampus. In addition, the results indicate that the P-CREB /BDNF signaling pathway may be an intermediate step in the neuroprotective role of crocin.

As a psycho-stimulant agent, MPH has a high potential for abuse and addiction (Morton and Stockton, 2000; Barrett et al., 2005; Martins et al., 2006; Berridge et al., 2006). According to the present study, chronic administration of MPH at a dose of 10 mg/kg was associated with an increase in escape latency and traveled distance in the MWM. These data suggest that MPH administration can disturb learning-related activity. In addition, on probe day, we found that MPH administration decreased the percentage of time spent that the rat spent in the target quarter of the MWM. These patterns suggest that chronic administration of MPH can deteriorate spatial memory – a result that confirms outcomes of a previous study showing that chronic administration of MPH was associated with a decline in learning and memory in rats (Scherer et al., 2010). MPH has been shown to cause a release of dopamine,

serotonin, and adrenaline in the brain, causing down-regulation of these amine receptors, which can consequently result in cognition impairment (Berridge et al., 2006). According to our results, high doses of crocin (40 and 80 mg/kg) can ameliorate MPH-induced cognition impairment. On the other hand, crocin alone can decrease escape latency and traveled distance, and also increase the presence of the animal in the target quarter in the MWM. Several previous studies have indicated that crocin and other similar herbal compounds can improve learning and memory (Hosseinzadeh et al., 2012). The results of the present study demonstrate that MPH at a dose of 10 mg/kg leads to a decrease in central square entry and time spent in the central square in the OFT, and can also increase ambulation distance and rearing number. According to these data, this dose of MPH can cause hyper-locomotion and result in the manifestation of depressive-like behavior. This dose of MPH likely causes a disturbance, specifically an increase, in motor activity (Kanazawa et al., 2017). On the other hand, our results show that crocin can decrease this type of depression in a dose-dependent manner, and can cause inhibition of MPH-induced hyper-locomotion and motor activity disturbance in the OFT. In addition, crocin alone can increase central square entry and the time spent in the central square in the OFT, and reduce ambulation distance and rearing number (Itzhak and Martin, 2002; Barbosa et al., 2011). Our results are consistent with a previous study demonstrating that a crocin-like derivative (i.e., quercetin) can alter manic-like behavior and hyper-locomotion induced by MPH (Kanazawa et al., 2017). Several previous studies have demonstrated that crocin can act as an antidepressant and modulate motor activity disturbances (Hosseinzadeh et al., 2004; Wang et al., 2010). It has been previously proposed that crocin can modulate cortical excitability, motor activity, and reaction speed in depressed individuals (Jam et al., 2017). A related study demonstrated that chronic administration of quercetin blocked MPH-stimulated hyper-locomotion in mice, which may reflect an antimanic-like effect (Kanazawa et al., 2017).

Our data indicate that MPH administration induces a rise in hippocampal MDA levels; however, crocin treatment (10, 20, 40, and 80 mg/kg) attenuated the MPH-induced increase in lipid peroxidation in the brain. The inhibitory influence of crocin on MDA levels was more pronounced at higher doses (40 and 80 mg/kg) in comparison to the lower doses (10 and 20 mg/kg) used in this study. Crocin alone (80 mg/kg) was also found to decrease MDA levels. These outcomes are similar to prior findings, which showed MPH-induced lipid peroxidation in the brain (Martins et al., 2006; Schmitz et al., 2012). According to these data, it

appears that part of the damaging effects of MPH are mediated by mitochondrial dysfunction, and crocin may play a role in moderating this process (Fagundes et al., 2010). Moreover, it has been indicated by prior work that crocin exerts neuroprotective properties by inhibiting the creation of free radicals in neurodegenerative diseases such as Alzheimer's disease (Hosseinzadeh et al., 2009; Khalili and Hamzeh, 2010; Rashedinia et al., 2015). In addition, the role of crocin as a scavenger of free radicals is apparent in this type of disorder (Hosseinzadeh et al., 2005; Naghizadeh et al., 2008). Our results showed that MPH (10 mg/kg) reduces the content of mitochondrial GSH, but increases levels of GSSG in hippocampal tissues. The conversion of GSH to GSSG by MPH is a main step that can start and trigger neurodegenerative signals in the brain (Martins et al., 2006; Fagundes et al., 2010), and this mechanism has damaging effects on the glutathione cycle and thus causes neural cell death (Martins et al., 2006; Fagundes et al., 2010). Furthermore, we found that several doses of crocin, particularly 40 and 80 mg/kg, increase GSH content while decreasing GSSG levels in animals treated with MPH (10 mg/kg). Crocin alone (80 mg/kg) was found to decrease GSSG levels and increase GSH levels. These results have been previously described by studies reporting that, by modulating the glutathione cycle, crocin can be therapeutically advantageous against neurodegenerative diseases as it stimulates GSH development (Ochiai et al., 2004a; 2004b).

In the present study, MPH administration reduced the activity of GPx, GR, and SOD in isolated hippocampal tissues. These results confirm prior reports that MPH abuse can reduce antioxidant defenses which may, in turn, result in neurodegeneration (Martins et al., 2006). It has been shown that GR is the key enzyme modulating the glutathione cycle (Hosseinzadeh et al., 2005). Thus, the MPH-induced reduction in GR activity results in an elevation in GSSG levels and a reduction in GSH levels, as observed in our results. Several recent reports demonstrate that MPH consumption leads to mitochondrial dysfunction and inhibits antioxidant enzyme activity in multiple cells, and these properties can cause MPH-induced degenerative effects on brain cells, such as in the hippocampus (Martins et al., 2006; Lagace et al., 2006). We found that crocin recovers the action of antioxidant enzymes in a dose-dependent manner. Crocin alone (80 mg/kg) was able to activate antioxidant enzymes. By stimulating GR, crocin enhances the conversion of GSSG to GSH and therefore, protects the brain against MPH-induced oxidative stress. Other experimental studies have also established that the observed anti-oxidative properties of crocin in neurodegenerative disorders and diseases are mediated by an increase in activity of GR and GPx (Bors

et al., 1984; Ochiai et al., 2004a). Moreover, a similar previous study demonstrated that prolonged quercetin treatment blocked the MPH-induced increase in oxidative stress (Kanazawa et al., 2017). Also, our results confirm prior studies showing a decrease in SOD action following MPH abuse (Lagace et al., 2006; Martins et al., 2006). Consistent with prior studies, crocin treatment was found to be effective in reversing the alcohol-induced decrease in SOD action in the hippocampal tissues (Ochiai et al., 2004a).

We confirmed that prolonged MPH administration significantly raises levels of pro-inflammatory cytokines such as IL-1 β and TNF- α in hippocampal tissue. We also found that, in high doses, crocin has robust potential for suppressing MPH-induced neuroinflammation, and this effect is dose-dependent. Further, treatment of the animals by crocin alone (80 mg/kg) was found to inhibit the inflammatory biomarkers IL-1 β and TNF- α . Our results are consistent with prior studies showing an increase in pro-inflammatory cytokines following abuse of MPH and other psychostimulants. It has been proposed that the MPH-induced increase in inflammation is a likely cause of the neurotoxic properties of MPH (Martins et al., 2006). Alternatively, crocin has been shown to have therapeutic potential for the management of neuroinflammation signaling cascades, thus protecting the brain against inflammation and related damage (Mehri et al., 2012).

Moreover, with respect to oxidative stress and inflammation, the present study confirms MPH-induced apoptosis in the hippocampus. According to the current study, administration of MPH increased the levels of the apoptotic protein, Bax, while simultaneously reducing levels of the anti-apoptotic protein, Bcl-2. These data are consistent with previous studies demonstrating that MPH abuse can cause brain impairment via triggering multiple apoptotic cascades (Andreazza et al., 2007; Bethancourt et al., 2009). At the same time, our results verified the anti-apoptotic effects of crocin against MPH administration, as shown by a reduction in Bax expression and an increase in Bcl-2 expression in the hippocampus. Indeed, we found that crocin alone (80 mg/kg) was able to decrease Bax levels and increase Bcl-2 levels. Previous studies confirmed that crocin treatment reduces cleaved caspase-3, the production of Bax, and nuclear condensation resulting from some neurodegenerative disorder and diseases (Oruc et al., 2016).

The anti-inflammatory, anti-apoptotic and anti-oxidative properties of crocin have been formerly described (Mehri et al., 2012; Rameshrad et al., 2018), and are consistent with our work; however, the involved signaling pathways remain unknown. To address this, we assessed the role of the P-CREB-BDNF signaling

pathway and aimed to identify the involved signaling pathway. Our data confirmed that administration of MPH ameliorates protein expression of CREB (total and phosphorylated) and BDNF in the hippocampus. In contrast, in high doses, crocin treatment enhances protein expression of CREB (total and phosphorylated) and BDNF. Therefore, these results indicate that crocin treatment restores the P-CREB-BDNF signaling cascade and protects the brain against MPH-induced neurotoxicity. As a transcription factor, P-CREB controls over a hundred target genes, particularly BDNF, which is involved in neuronal regeneration, development, survival, excitability, addiction, depression, and cognition (Carlezon et al., 2005). Furthermore, dysregulation of the CREB transcriptional cascade has been found to prompt oxidative stress, apoptosis, and neurodegeneration (Mayr and Montminy, 2001; Carlezon et al., 2005). Previous molecular studies have consistently reported that the phosphorylated form of CREB plays a key role in various herbal and chemical neuroprotective processes (Mayr and Montminy, 2001). Based on the results of numerous studies, P-CREB (the activated form of CREB) leads to the creation of BDNF, which is a ligand of the TrkB receptor. These studies demonstrate that, by stimulating its own receptor (TrkB), BDNF can protect brain cells from degeneration and can promote the survival of neurons (Martinowich et al., 2003). In the present study, it appears that a reduction of P-CREB protein levels, via MPH, can affect the aforementioned BDNF/TrkB signaling cascade, which triggers neurodegeneration, apoptosis, inflammation, and oxidative stress. Administration of crocin, in contrast, can inhibit the reported toxic actions of MPH and trigger the cascade of P-CREB/BDNF/TrkB. Indeed, administration of crocin alone (80 mg/kg) activates expression of both P-CREB and BDNF. As our data demonstrate, crocin alone can modulate cognition and motor activity and also inhibit the occurrence of oxidative stress, inflammation, and apoptosis. Thus, it seems that crocin has neuroprotective properties and its administration alone not only has no adverse effects, but has protective and beneficial molecular and behavioral effects. The initial aim of this experimental group (i.e., treatment of crocin alone in high dose) was to test the safety of crocin after a longer period of time. As noted, for all parameters, we found that crocin treatment alone had beneficial molecular and biochemical effects. Consistent with our results, it has been shown that the P-CREB-BDNF signaling pathway is associated with a modification of numerous functions in the brain, such as learning, memory, mood balances, and reward mechanisms (Martinowich et al., 2003; Yoshii and Constantine-Paton, 2010; Cao et al., 2013; Razavi et al., 2017).

CONCLUSION

The results of the present study demonstrate that treatment with crocin – one of the main compounds of saffron – can reduce MPH-induced cognitive impairment, hyper-locomotion, and can inhibit apoptosis, oxidative stress, and inflammation in rats. Based on these data, crocin may act as a neuroprotective mediator against the reported adverse neurobehavioral and neurochemical effects of MPH. According to our study, crocin has neuroprotective properties in this manner, potentially via stimulation of the P-CREB-BDNF signaling pathway, and this concept was summarized in Fig. 5. Via the aforementioned neuroprotective properties, crocin could be a candidate treatment approach for inhibiting the adverse effects of drug abuse in humans. Indeed, crocin may act as neuroprotective and adjunct agent against the adverse neurobehavioral and neurochemical effects of drug abuse in humans.

LIMITATION OF THE STUDY

One limitation of the current study was that we did not evaluate the side effects of crocin at high doses, because the primary goal of this study was to introduce its neuroprotective effects against MPH-induced sequelae. An evaluation of potential side effects of crocin treatment was not the aim of our research. However, as mentioned previously, our studies have demonstrated that crocin in high doses (80 mg/kg) can not only affect the side effects on behavioral and molecular aspects, but also had protective effects in the rat hippocampus.

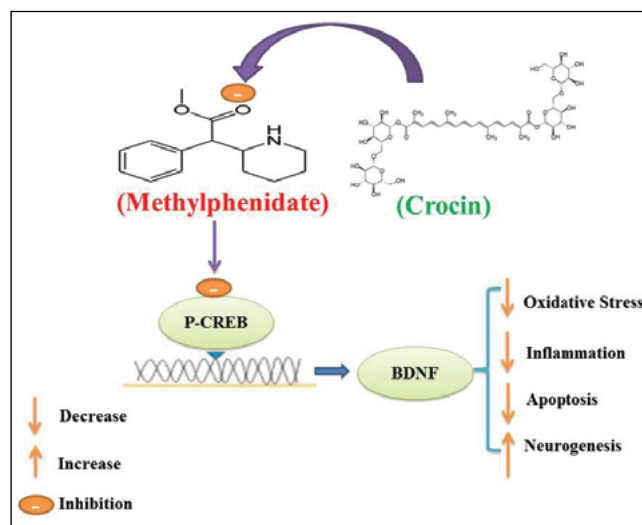


Fig. 5. Possible role of CREB-BDNF signaling pathway in crocin antioxidant, anti-inflammatory and anti-apoptotic effects against methylphenidate-induced neurochemical sequelae.

Also, the condition and effects of MPH dependency in human and animals are different and cannot be extrapolated to human beings. We suggest that further studies should evaluate the translational relevance in humans, as well as, dosage and toxicity of both MPH and crocin, to better evaluate the effects and possible side effects.

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