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Original Paper

Pretreatment with Crocin, Carvacrol, and Physical Exercise and Attenuating Motor and Memory Impairments in Hemiparkinsonian Rats by Anti-inflammatory and Antioxidant Mechanisms

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Background: Parkinson Disease (PD) is a chronic neurodegenerative disorder caused mainly by the degeneration of dopaminergic neurons in the substantia nigra leading to motor dysfunctions. Non-motor symptoms, including memory impairments, follow the motor symptoms in Parkinson patients. Male Wistar rats ran on a horizontal treadmill and or pretreated with crocin at a dose of 100 mg/kg and carvacrol at a dose of 25 mg/kg for a week. Then, 16 µg 6-hydroxydopamine (6-OHDA), a neurotoxin, was microinjected into the left medial forebrain bundle, and treatments continued for six more weeks. Aversive memory, rotational behavior, inflammatory, and oxidative stress parameters were assessed 6 weeks after surgery.

Results: The results showed that pretreatment with crocin alone and in combination with exercise decreased the total number of rotations compared with the 6-OHDA-lesioned group. Furthermore, treatment with crocin along with exercise training improved aversive memory. The crocin and exercise (alone and in combination) reduced tumor necrosis factor-alpha levels in the striatum. Moreover, treatment with crocin decreased the lipid peroxidation levels in the hippocampus, and exercise training increased the total thiol concentration. Treatment with carvacrol and exercise reduced rotational behavior and improved aversive memory deficit, accompanied by decreased lipid peroxidation levels and increased total thiol concentration in the striatum and or hippocampus.

Conclusion: In conclusion, our findings indicated that pretreatment with crocin as a carotenoid and carvacrol as a phenol along with treadmill exercise ameliorated motor and memory deficits induced by 6-OHDA, which is considered to be due to their antioxidant and anti-inflammatory activities. The results suggest that combined therapy with crocin and exercise or carvacrol and exercise may protect from motor and memory deficits in patients with Parkinson.

Keywords: Exercise, Memory, Motor activity, Oxidative stress, Parkinson disease

Introduction

Parkinson's disease (PD) is a chronic neurodegenerative disorder, which is caused mainly by the degeneration of dopaminergic neurons in the substantia nigra, leading to motor dysfunctions such as resting tremor, muscle rigidity and bradykinesia (Fahn, 2003). In addition to motor dysfunctions, cognitive deficits such as learning and memory impairments and dementia are seen in a high percentage of PD patients (Brown and Marsden, 1984). The etiology of the disease is not well-known

but, various factors such as oxidative stress (Dias et al., 2013), mitochondrial dysfunction (Subramaniam and Chesselet, 2013) and neuroinflammation (Zhang et al., 2018) are effective factors in the onset of this disease. Among these factors, oxidative stress plays an important role in the degeneration of dopaminergic neurons in PD. Based on the oxidative stress hypothesis, imbalance in oxidant and antioxidant agents leads to oxidative stress (Dias et al., 2013). During this process, enhancement of the production of reactive oxygen species (ROS) leads to neuronal damage and death through membrane lipid peroxidation, protein oxidation, and DNA damage (Sayre et al., 2008).

There is also evidence suggesting that inflammation plays a pathogenic role in PD (Hartmann et al., 2003). Immunohistochemical studies have demonstrated the presence of activated microglia, increased cytokine expression, and upregulation of inflammatory-associated factors in the striatum, substantia nigra and cerebrospinal fluid of PD patients (Olanow,

2007, Nagatsu et al., 2000). Evidence has indicated that the levels of several cytokines, including tumor necrosis factor (TNF- α) are significantly elevated in the substantia nigra and striatum (Pieper et al., 2008, Ros-Bernal et al., 2011). TNF can activate the abundant numbers of microglia in the midbrain, potentiating inflammatory responses that lead to auto amplification of ROS, nitric oxide, and superoxide radicals and eventually oxidative damage of dopaminergic neurons (Niranjan, 2014).

Despite many advances in the treatment of PD, most drugs such as; L-DOPA only control disease symptoms and do not stop or delay the degeneration of dopaminergic neurons (De Lau and Breteler, 2006). Therefore, it has been suggested that antioxidant molecules such as carotenoids and polyphenols and compounds that interfere with the production of reactive oxygen species may have neuroprotective effects. Crocin possesses several pharmacological effects, including; antioxidant (Chen et al., 2008), anti-inflammatory (Nam et al., 2010), cancer cell proliferation inhibitor (Magesh et al., 2006). It has also been reported that crocin protects against oxidative damage to the brain vessels (Zheng et al., 2007), kidney tissues (Hosseinzadeh et al., 2005), heart (Goyal et al., 2010) and retina (Yamauchi et al., 2011) under different experimental conditions. Carvacrol (CAR, 2-methyl-5-isopropyl phenol) is a phenolic monoterpene abundantly present in the essential oil of the family Lamiaceae. Carvacrol has been reported to have many pharmacological benefits, including antibacterial, antifungal, antioxidant, antinociceptive, anti-inflammatory and anticancer (Can Baser, 2008). Moreover, exercise and physical activity are among the non-pharmacological methods used to prevent neurodegenerative disorders. Although exercise alone has significant neuroprotective properties, the benefits of exercise and its neuroprotective effects in PD needs further research. A meta-analysis demonstrated that exercise might improve physical functions, life quality, balance, and gait speed in PD patients (Goodwin et al., 2008), nevertheless, its neuroprotective effects in PD have been challenged. As reported, performing treadmill exercise for 6 weeks in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced mouse model of PD, does not prevent mitochondrial inhibition (Aguiar et al., 2014) or nigrostriatal neurodegeneration (Gerecke et al., 2012). This means that the neuroprotective effects of exercise, and the mechanism by which treadmill exercise exerts its effects in PD, are not completely understood. The present study was undertaken to examine the neuroprotective effects of crocin as a carotenoid at a dose of 100 mg/kg, carvacrol as a phenol at a dose of 25 mg/kg and treadmill exercise (alone and in combination) in a 6-OHDA model of PD in rat. For this purpose, the effects of exercise, crocin and carvacrol on motor activity (as assessed by rotational behavior), aversive memory, striatal and hippocampal inflammatory biomarker (TNFα) and oxidative stress status (by measuring lipid peroxidation levels and total thiol concentration) were examined.

Methods

Experimental animals

The experimental animals used in the present study were adult male Wistar rats weighing 250–350 g. They were maintained at a controlled temperature (22 ± 2°C) with a 12-hour dark/light cycle and had free access to water and food. The Ethics Committee for Animal Experiments at Isfahan University of Medical Sciences approved the study (Approval No. 194036) and all experiments were conducted under the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publication, 8th edition, 2011).

Experimental design

The animals were randomly assigned into 9 groups, including; 1) Normal saline sham-operated group (injection of ascorbate-saline 0.2% into the left medial forebrain bundle (MFB), saline ip, n = 7), 2) sham-operated group (injection of 0.2% ascorbate-saline into the left medial forebrain bundle (MFB), 1% Tween 80 ip, n = 7) 3) 6-OHDA-lesioned group (injection of 16 µg 6-OHDA into left MFB, saline ip, n = 7), 4) 6-OHDA-lesioned group (16 µg 6-OHDA into the MFB, 1% Tween 80 ip, n = 7), 5) crocin group (injection of 16 µg 6-OHDA into left MFB, 100 mg/kg crocin ip, n = 7), 6) Carvacrol group (16 µg 6-OHDA into the MFB, 25 mg/kg carvacrol ip, n = 10), 7) exercise group (injection of 16 µg 6-OHDA into left MFB, treadmill exercise, n = 8) ,8) crocin+exercise group (injection of 16 µg 6-OHDA into left MFB, 100 mg/kg crocin along with treadmill exercise, n = 8). 9) carvacrol + exercise group (injection of 16 µg 6-OHDA into the left MFB, 25 mg/kg carvacrol and treadmill exercise, n = 7). Carvacrol was emulsified with 1% Tween 80 and dissolved in normal saline. It should be noted that the injection of crocin (100 mg/kg, ip), carvacrol (25 mg/kg, ip), normal saline or 1% Tween 80 and treadmill exercise started one week before the 6-OHDA or ascorbate-saline injection and continued for 6 weeks after surgery.

Surgery and 6-OHDA lesion

Under chloral hydrate (450 mg/kg, ip) anesthesia (Rajaei et al., 2005), rats were positioned in the stereotaxic apparatus (Stoelting, USA). The scalp was cleaned with an iodine solution and lidocaine was injected (2% solution, Sc). A midline skin incision was made with subsequent drilling of the skull. The 6-OHDA compound (16µg/4µl 0.2% ascorbate-saline)(Rajaei et al., 2005) was injected into the left MFB by a Hamilton microsyringe according to the coordinates:

AP: -3.6 mm; ML: -1.8 mm; DV: -8.2 mm12. The rats in the sham-operated group also received ascorbate-saline and 1% Tween 80 dissolved in normal saline at the same volume as the treated groups. The injection rate was 1 µl/min and the needle was kept in place for a further five minutes after injection for complete absorption of the toxin. After surgery, the rats were placed singly into a clean page and kept war until recovery from anesthesia was complete.

Treadmill training

A five-lane motorized rat treadmill was used for exercise training. One week before the essential protocol, the exercised groups of animals were trained on the treadmill, running for 5 days/week, 5 min/day with a speed of 10 m/min to reduce their stress to the new environment (Tuon et al., 2012). One week before surgery, the animals in the exercised group were trained on the treadmill running at 5 days/week, 40 min/day with a speed up to 17 m/min (5 min at 7 m/min, 30 min at 17 m/min, 5 min at 7 m/min). This protocol continued for six weeks after surgery. The untrained animals were placed on the switched-off treadmill for the same weeks as the exercise groups.

Passive avoidance memory

Passive avoidance learning was assessed by a shuttle box at the end of week 6. The apparatus consisted of a light and a dark compartment, connected by a guillotine door. In the training session, animals were placed individually in the light compartment for one minute. After the opening of the door and the movement of the rat into the dark chamber, the door was closed and a 0.5 mA foot electric shock was delivered through the grid floor for three seconds. In the test session, each rat was again placed into the light compartment. The step-through latency to entering the dark compartment was measured as a positive index of memory performance, with a 300 second cut-off time (Rajaei et al., 2016).

Apomorphine-induced rotations

The hemiparkinsonian rats were diagnosed by observing the rotational behavior after an injection of apomorphine hydrochloride (Sigma-Aldrich, USA) at the end of the 2nd and 6th week after surgery. Apomorphine hydrochloride was dissolved in normal saline and injected intraperitoneally at a dose of 2 mg/kg. On the test day, the animals were allowed to habituate to a transparent plexiglass container (28×28×50 cm) for 10 minutes. One minute after the injection of apomorphine, full rotations were counted at 10 minute intervals for 30 minutes in a dimly-lit, quiet room. Injection of the neurotoxin 6-OHDA may not be accurate, and some surgical animals did not show rotation after apomorphine injection and were therefore excluded. The number of ipsilateral rotations was counted as positive scores and those of contralateral rotations as negative scores. The net number of rotations was defined as the difference between the rotations in both directions (Fujita et al., 1996).

Cytokine levels

After centrifugation of striatal homogenates at 3000 rpm for 5 min, the supernatants were assayed for TNF-α and IL-10 using commercially available ELISA kits (ebioscience Co, San Diego, CA, USA) according to the manufacturer's instructions. Results were shown as pg/ml.

Lipid peroxidation levels

The lipid peroxidation level of the hippocampus and/or striatum was measured as malondialdehyde, which reacts with thiobarbituric acid as a thiobarbituric acid reactive substance (TBARS) to produce a red-colored complex that has a peak absorbance (A) at 535 nm. A mixture of trichloroacetic acid, thiobarbituric acid, and HCl was added to 1 ml of homogenate, and the mixture was heated for 45 minutes in a boiling water bath. After cooling and centrifugation at 1000 g for 10 minutes, the absorbance was measured at 535 nm. The level of TBARS was calculated by: C (M) = absorbance/1.65 × 105 (Ahmadi et al., 2017) .

Total thiol concentration

Total sulfhydryl groups were measured using 2.2′-Dinitro-5.5′-dithiodibenzoic acid (DTNB) as the reagent. This reagent reacts with the sulfhydryl groups to produce a yellow- colored complex that has a peak absorbance at 412 nm. Briefly, 1 ml tris-EDTA buffer was added to 50 µl homogenate 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 (minutes), 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: (A2-A1-B) × 1.07/0.05 × 13.6 (Ahmadi et al., 2017).

Histology

The brains were removed and stored in 10% formalin for 72 hours. 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 the injection site of 6-OHDA was determined by reference to a rat brain atlas (Paxinos, 2005).

Statistical analysis

The results are presented as mean ± SEM. Data were analyzed by one-way ANOVA followed by Tukey's *post hoc* test. Results were considered significant at $P < 0.05$.

Results

Apomorphine-Induced Rotations

Administration of apomorphine to 6-OHDA-lesioned rats produced contralateral rotations towards the lesion side at the end of the sixth week after surgery, indicating unilateral damage to the left striatum ($P<0.001$, Figure 1a). Pretreatment with crocin alone and along with treadmill exercise reduced the total net number of rotations as compared with the 6-OHDA-lesioned group ($P<0.01$, $P<0.01$, respectively). However, treadmill exercise alone did not change rotations as compared with the 6-OHDA-lesioned group (Figure 1a).

Pretreatment with carvacrol and treadmill exercise reduced the contralateral rotations compared with the 6-OHDA-lesioned group ($P<0.05$). However, pretreatment with carvacrol at a dose of 25 mg/kg and treadmill exercise alone did not change rotations compared with the 6-OHDA-lesioned group (Figure 1b).

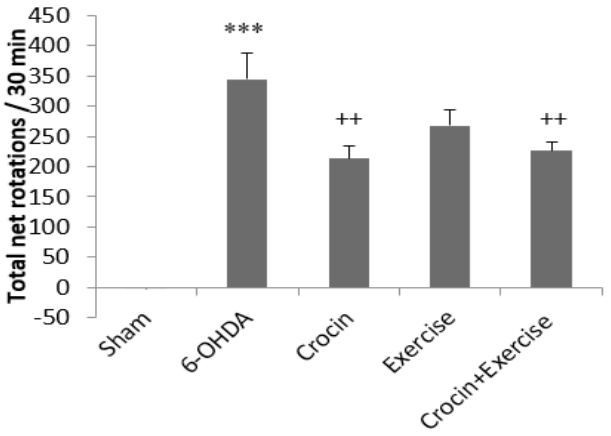

Figure 1a: The total net number of rotations (mean±SEM) induced by apomorphine (2 mg/kg, IP) for 30 min among the experimental groups at the end of week 6. Crocin was administered daily at a dose of 100 mg/kg for 7 weeks (n=7-8 for each group). $^{***}P<0.001$ vs sham group, $^{++}P<0.01$ vs 6-OHDA-lesioned group.

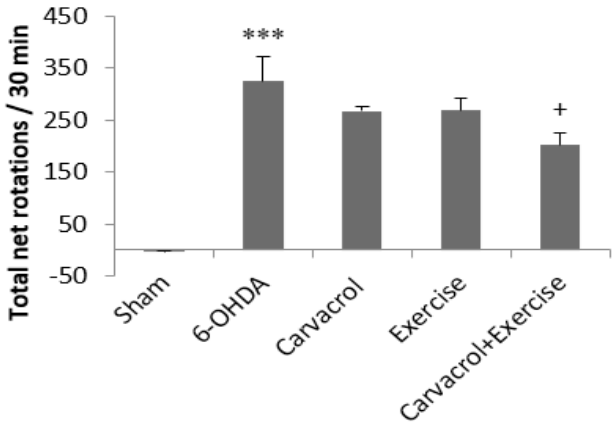

Figure 1b: Effects of carvacrol and treadmill exercise on apomorphine-induced rotations (mean±SEM) among the experimental groups at the end of week 6. Carvacrol was administered daily at a dose of 25 mg/kg for seven weeks (n=7-10 for each group). ***P<0.001 vs sham group, +P<0.05 vs 6-OHDA-lesioned group.

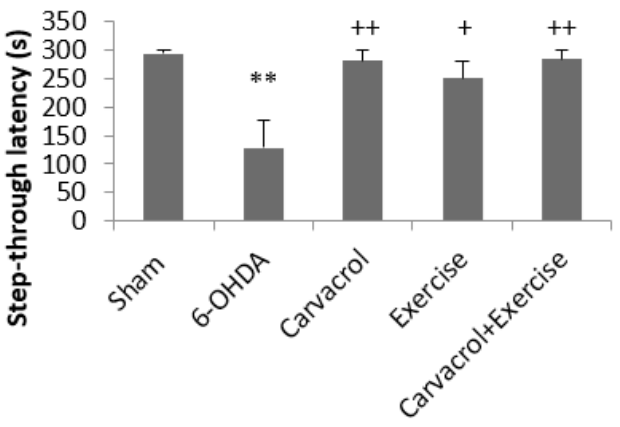

Figure 2b: Effects of carvacrol and treadmill exercise on step-through latency (mean±SEM) among the experimental groups at the end of week 6. The passive avoidance test was used to assess aversive memory. Carvacrol was administered daily at a dose of 25 mg/kg for 7 weeks (n=7-10 for each group). **P<0.01 vs sham group, +P<0.05, ++P<0.01 vs 6-OHDA-lesioned group.

Passive Avoidance Memory

As shown in Figure 2a, the step-through latency of 6-OHDA-lesioned rats was shorter than sham group rats at the end of week 6 (P<0.05, Figure 2a). Moreover, pretreatment with crocin along with treadmill exercise significantly increased the latency as compared with the 6-OHDA-lesioned group (P<0.05). Treatment with crocin and treadmill exercise alone did not significantly change the latencies compared with the 6-OHDA-lesioned group, though there was a tendency toward an increase in latencies in both groups (Figure 2a). As shown in Figure 2b, the step-through latency of 6-OHDA-lesioned rats was shorter than sham group rats at the end of week 6 (P<0.01, Figure 2b). Moreover, pretreatment with carvacrol at a dose of 25 mg/kg (P<0.01) or treadmill exercise (P<0.05) and in combination (P<0.01) significantly increased the latencies compared with the 6-OHDA-lesioned group (Figure 2b).

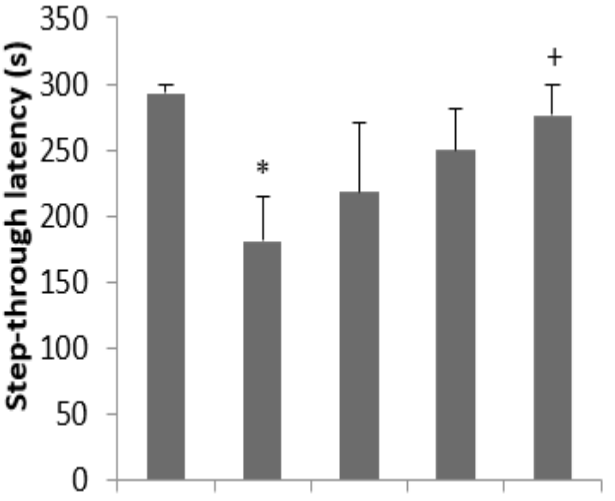

Figure 2a: Step-through latency (mean±SEM) among the experimental groups at the end of week 6. Crocin was administered daily at a dose of 100 mg/kg for 7 weeks (n=7-8 for each group). *P<0.05 vs sham group, +P<0.05 vs 6-OHDA-lesioned group.

TNF-α Levels

A significant increase in the TNF-α levels was found in the striatum of 6-OHDA-lesioned rats (P<0.01, Figure 3) compared with the sham group. In addition, crocin at a dose of 100 mg/kg and treadmill exercise, alone (P<0.01, P<0.001, respectively) and in combination (P<0.01) reduced the TNF-α levels in the striatum at the end of the week 6, compared with the 6-OHDA-lesioned group (Figure 3).

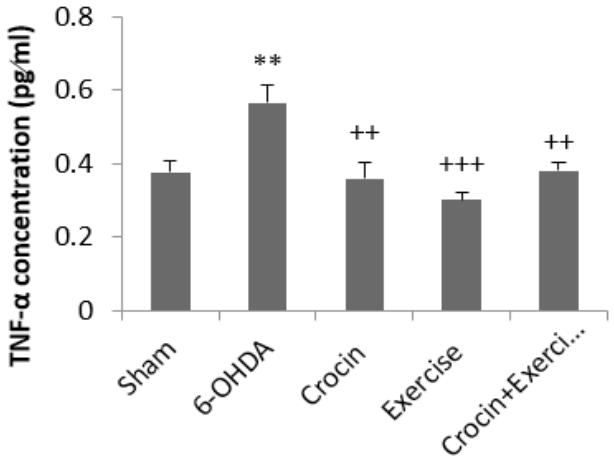

Figure 3: TNF-α levels (mean±SEM) in the striatum among the experimental groups at the end of week 6. Crocin was administered daily at a dose of 100 mg/kg for 7 weeks (n=7-8 for each group). **P<0.01 vs sham group, ++P<0.01, +++P<0.001 vs 6-OHDA-lesioned group.

Lipid Peroxidation Levels

The degree of free radical damage following MFB lesion was assessed using lipid peroxidation and measured as TBARS levels. A significant increase in the levels of TBARS was found in the hippocampus of 6-OHDA-lesioned rats (P<0.05, Figure 4a) compared with the sham group. Moreover, pretreatment of lesioned rats with crocin at a dose of 100 mg/kg reduced the TBARS levels in the hippocampus at the end of week 6 (P<0.05). However, treatment with crocin along with treadmill exercise and exercise alone did not change the TBARS levels in

the hippocampus at the end of week 6 (Figure 4a). A significant increase in the levels of TBARS was found in the striatum (P<0.05) of 6-OHDA-lesioned rats compared with the sham group (Figure 4b). Moreover, treadmill exercise alone and in combination with carvacrol decreased TBARS levels in the striatum (P<0.05, P<0.05) at the end of week 6 (Figure 4b).

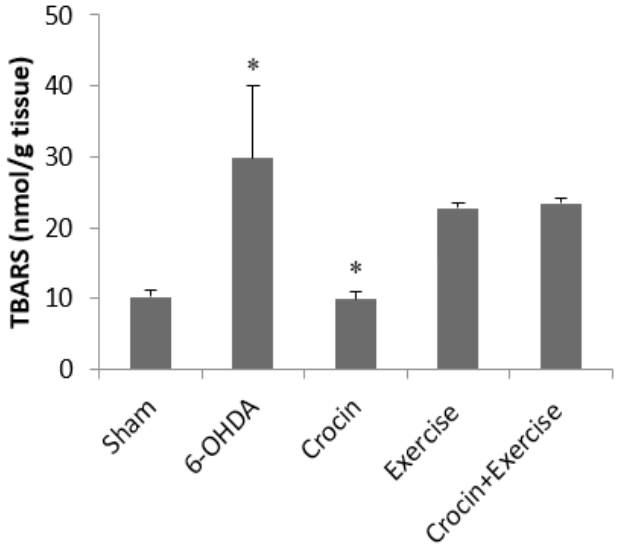

Figure 4a: Lipid peroxidation levels (mean±SEM) in the hippocampus among the experimental groups at the end of week 6. Crocin was administered daily at a dose of 100 mg/kg for 7 weeks (n=7-8 for each group). *P<0.05 vs sham group, +P<0.05 vs 6-OHDA-lesioned group.

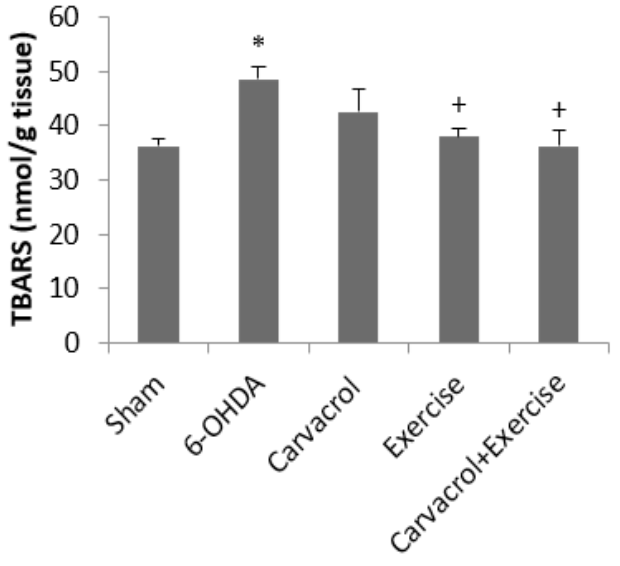

Figure 4b: The effects of carvacrol and treadmill exercise on TBARS levels (mean±SEM), an index of lipid peroxidation, in the striatum among the experimental groups at the end of week 6. Carvacrol was administered daily at a dose of 25 mg/kg for 7 weeks (n=7-10 for each group). *P<0.05 vs sham group, +P<0.05 vs 6-OHDA-lesioned group.

Total Thiol Concentration

Statistical analysis showed the total thiol concentration in the hippocampus significantly decreased in the 6-OHDA-lesioned group compared with the sham group (P<0.01, Figure 5a). Pretreatment with crocin and treadmill exercise and exercise alone significantly increased total thiol concentration compared with the 6-OHDA-lesioned group (P<0.01). However, treatment with crocin at a dose of 100 mg/kg alone did not significantly change the total thiol concentration compared with the 6-OHDA-lesioned group (Figure 5a). Statistical analysis showed no significant change in total thiol concentrations in the hippocampus of the sham and experimental groups at the end of week 6 (Figure 5b).

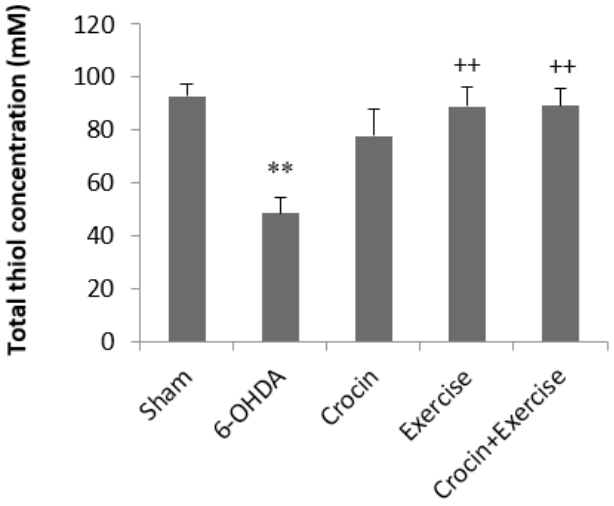

Figure 5a: Total thiol concentration (mean±SEM) in the hippocampus among the experimental groups at the end of week 6. Crocin was administered daily at a dose of 100 mg/kg for 7 weeks (n=7-8 for each group). **P<0.01 vs sham group, ++P<0.01 vs 6-OHDA-lesioned group.

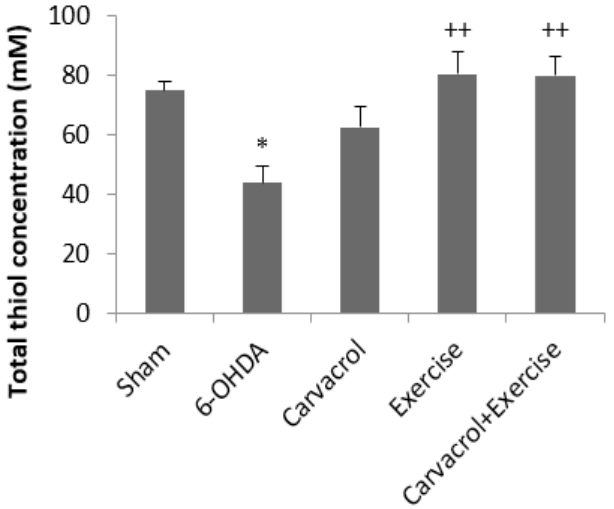

Figure 5b: Effects of carvacrol and treadmill exercise on total thiol concentration (mean±SEM), an index of antioxidant potential, among the experimental groups at the end of week 6. Carvacrol was administered daily at a dose of 25 mg/kg for 7 weeks (n=7-10 for each group). *P<0.05 vs sham group, ++P<0.01 vs 6-OHDA-lesioned group.

Discussion

Parkinson Disease (PD) is a heterogeneous neurodegenerative disorder characterized by loss of dopaminergic nigrostriatal neurons. Studies have shown that PD is a multifactorial disease, and several factors, such as oxidative stress, mitochondrial dysfunction, and neuroinflammation, are effective factors in the onset and progression of this disease. The 6-OHDA compound is a dopaminergic neurotoxin that undergoes auto-oxidation and produces cytotoxic hydrogen peroxide, a reactive oxygen species. The increase in reactive oxygen species levels causes abnormalities in cell structure and metabolism and eventually leads to neuronal degeneration. Our results showed that microinjection of 6-OHDA into MFB increased TNF-α levels in the striatum. This finding is in line with previous studies that have reported the increased levels of cytokines, in particular TNF-α, in the substantia nigra and striatum of 6-OHDA-lesioned rats. Pharmacological evidence implicates TNF-dependent events in the death of dopaminergic neurons. For instance, it has been reported that inhibition of soluble TNF signaling for 2 weeks with a dominant negative TNF inhibitor attenuated 6-OHDA-induced dopaminergic neuron loss. Interestingly, a 6-OHDA lesion also results in an inflammatory response that can only be controlled approximately 12 days after the 6-OHDA lesion. Microgliosis and increased pro-inflammatory mediators in the brain are found in the 6-OHDA PD model. In the present study, the microinjection of 6-OHDA into the left MFB resulted in motor deficits observed by increased rotations. The unilateral lesion of the nigrostriatal dopaminergic system by 6-OHDA decreases dopamine levels in the striatum and upregulates dopamine postsynaptic receptors on the same side. These changes produce a motor asymmetry that can be evaluated by dopamine agonists such as apomorphine . Apomorphine-induced rotations in the 6-OHDA-lesioned rats are a reliable marker for nigrostriatal dopamine depletion. Our findings also showed a significant decrease of the apomorphine-induced rotations in the crocin group compared with the 6-OHDA group. The improvement effect of crocin on motor deficits could be partly attributed to the anti-inflammatory effect of crocin. As shown in the results, crocin reduced TNF-α levels in the striatum. To our knowledge, this is the first study reporting the anti-inflammatory effects of crocin by reducing TNF-α levels in a 6-OHDA model of PD. Likewise, it has been reported that crocin reduced the LPS-stimulated production of TNF-α, interleukin-1β, and intracellular reactive oxygen species from activated microglia. Moreover, anti-inflammatory effects of crocin by reducing TNF-α have been reported in other inflammatory insults, such as rheumatoid arthritis and hemorrhagic shock. Accordingly, we can conclude that the neuroprotective effect of crocin in the striatum of parkinsonian rats could be partly due to its anti-inflammatory activity. Our data also showed that treadmill exercise reduced TNF-α levels in the striatum of 6-OHDA-lesioned rats, supporting reports that show anti-inflammatory effects of exercise in the PD model. For instance, Tuon et al. have reported that 8 weeks of treadmill exercise improved motor deficits and decreased TNF-α and IL-1ß expression in the substantia nigra pars compacta and striatum in MPTP-induced mouse model of PD. Behavioral analysis in the present study showed that treadmill training for 7 weeks did not significantly attenuate the apomorphine-induced rotations in hemiparkinsonian rats. However, there was a tendency toward a decrease in rotations in the exercise group (Figure 1), suggesting that exercise could have a modest neuroprotective effect against this neurotoxin insult. In this context, several studies have shown that treadmill exercise improved motor deficits in the 6-OHDA-lesioned rats, while others have reported that treadmill training does not ameliorate locomotor deficits in the 6-OHDA model of PD. This discrepancy in the experimental results could be due to differences in the experimental method, including the severity of the nigrostriatal lesion, as well as the duration and intensity of the applied exercise regimen. For instance, Petzinger et al. have shown that the degree of nigrostriatal neuron lesion could alter the influence of exercise on dopamine level. Furthermore, Lau et al. reported that exercise performance 1 week before, 5 weeks during, and 12 weeks (a total of 18 weeks) after injury improved motor activity. According to our results, treatment with crocin along with exercise significantly reduced the number of rotations as compared with the 6-OHDA-lesioned group. Altogether, improvement of motor activity in this group could be due to anti-inflammatory effects of crocin and exercise in the 6-OHDA model of PD. The present study also examined the effect of carvacrol, as an antioxidant agent, in a 6-OHDA model of PD. Our results showed that treatment with carvacrol at a dose of 25 mg/kg did not decrease the apomorphine-induced rotations and did not change the oxidative stress biomarker in the striatum of PD rats. This finding is inconsistent with a recent study that reported the neuroprotective effect of carvacrol at a single dose of 40 mg/kg upon the neurodegeneration induced by 6-OHDA intrastriatal injections in mice. The reasons for this discrepancy could be related to the extent of the lesion, dosage of carvacrol and duration of treatment. We used a chronic 6-OHDA-induced rat model of PD with severe neurodegeneration. The initial oxidative stress caused by 6-OHDA may be attenuated by the antioxidant activity of carvacrol. However, when there is substantial ongoing oxidative stress and neurodegeneration, the antioxidant response wanes or is overwhelmed over time and, at that point, carvacrol cannot act as an antioxidant.

Our results also showed that exercise, 1 week before and 6 weeks after 6-OHDA injection, reduced lipid peroxidation levels in the striatum. Similar to this result, it has been reported that treadmill exercise reduced the level of striatal carbonylated proteins and increased the superoxide dismutase levels in the striatum of mice with PD. It has also been shown that aerobic exercise for eight weeks increased the level of antioxidant enzymes, such as superoxide dismutase and catalase, and reduced oxidative damage to lipids and proteins in the striatum of hemiparkinsonian rats. However, our findings also showed that treatment with carvacrol plus treadmill exercises significantly decreased the apomorphine-induced rotations, accompanied by decreased TBARS levels with exercise. Besides, the carvacrol at a dose of 25 mg/kg might increase the total thiol concentration in the striatum. So the positive effects of carvacrol and exercise on improving motor behavior could partly be due to their synergism effects on the redox system in the striatum. In addition to motor deficits, PD patients also suffer from cognitive impairments. Cognitive impairments gradually occur due to the spread of damage to the other parts of the brain. 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 essential role in memory impairment. In our

study, passive avoidance was used to examine whether crocin, carvacrol, and treadmill exercise could improve the aversive memory of parkinsonian rats. The task is based on the motivation of passive avoidance from the fear of foot shock. Our results showed that treatment with crocin along with exercise training improved aversive memory deficits induced by 6-OHDA. However, crocin treatment and exercise alone partially improved memory deficits. Memory-enhancing effects of these agents could be due to their synergistic effects on the redox system in the hippocampus.

As shown in the results section, crocin at a dose of 100 mg/kg significantly reduced TBARS levels in the hippocampus. Also, treadmill exercise for 7 weeks increased total thiol concentration, as an index of total antioxidant potential, in the hippocampus. In agreement with this, it has been reported that aerobic exercise for 4 weeks increased the level of antioxidant enzymes, such as superoxide dismutase and catalase, in the hippocampus of young rats. Also, crocin possesses remarkable radical scavenging activity (Assimopoulou et al., 2005), and its antioxidant effects stronger than those of alpha-tocopherol . Therefore, the beneficial effect of crocin along with exercise on memory function could be partly due to its antioxidant activity so that it can overcome the destructive effects of 6-OHDA and improve memory deficits. As seen in the results, treatment with carvacrol significantly improved memory impairment in parkinsonian rats. In line with this, it has been reported that carvacrol (25, 50, 100 mg/kg) attenuated diabetes-associated cognitive deficits by decreasing malondialdehyde levels and increasing superoxide dismutase activity and, reduced glutathione levels in the hippocampus and cortex of diabetic rats. A recent study reported that chronic treatment with carvacrol improved passive avoidance memory in a rat model of PD; however, the mechanism of action was not examined (Haddadi et al., 2018). Biochemical data in the present study did not confirm the antioxidant activity of carvacrol in the hippocampus. The mechanism by which carvacrol improves memory deficits in PD could be due to anticholinesterase and anti-inflammatory activities of carvacrol.

In conclusion, our findings indicated that pretreatment with crocin (as a carotenoid) and treadmill exercise (alone and in combination) reduced inflammation in the striatum and oxidative stress in the hippocampus of hemiparkinsonian rats and ameliorated motor and memory deficits induced by 6-OHDA. Moreover, long-term treatment with carvacrol (as a phenol) and treadmill exercise ameliorated motor and memory deficits by modulating oxidative stress in the striatum and hippocampus of hemiparkinsonian rats. In general, the results suggest that combined therapy with crocin and exercise or carvacrol and exercise may be protective for motor and memory deficits in PD patients.

Acknowledgments

It is not a case

Ethical Permission

All principles of working with laboratory animals were observed based on the protocol of keeping and working with laboratory animals approved by Isfahan University of Medical Sciences. The animal was under general anesthesia throughout the surgery. This study also has the code of ethics from Isfahan University of Medical Sciences No. IR.MUI.REC.1395.3.676.

Conflict of interest

The authors declared no conflict of interest.

Authors' Contribution

Somayeh Shahidani, Leila Hamzehloei: performing project
Ziba Rajaei: Project designer
Hojjatallah Alaei: Project consultant

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