

Chronic treatment with carvacrol improves passive avoidance memory in a rat model of Parkinson's disease

O tratamento com carvacrol melhora a memória de esquiwa passiva em um modelo da doença de Parkinson em ratos

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ABSTRACT

The present study investigated the effects of carvacrol on motor and memory deficits as well as hyperalgesia in the 6-OHDA-lesioned rat model of Parkinson's disease. The animals were subjected to unilateral microinjection of 6-OHDA into the medial forebrain bundle and treated with carvacrol (25, 50 and 100 mg/kg, ip) for six weeks after surgery. The 6-OHDA-lesioned rats showed contralateral rotations towards the lesion side, which was accompanied by learning and memory deficits in a passive avoidance test and a decrease in tail withdrawal latency in a tail flick test at the end of week 6. The results also showed that treatment with carvacrol at a dose of 25 mg/kg ameliorated memory deficits, with no effect on rotations and hyperalgesia in lesioned rats. In conclusion, carvacrol improves memory impairments in rats with Parkinson's disease; therefore, it may serve as an adjunct therapy for the alleviation of memory deficits in Parkinson's disease patients.

Keywords: Carvacrol; 6-hydroxydopamine; memory; motor activity; hyperalgesia; Parkinson's disease.

RESUMO

O presente estudo investigou os efeitos do carvacrol nos déficits motores e de memória, bem como na hiperalgesia, em um modelo da doença de Parkinson (DP) em ratos com lesões 6-OHDA. Os animais foram submetidos a microinjeção unilateral de 6-OHDA no feixe mediano do prosencéfalo e tratados com carvacrol (25, 50 e 100 mg / kg, ip) durante 6 semanas após a cirurgia. Os ratos com lesões 6-OHDA mostraram rotações contralaterais para o lado da lesão, que foram acompanhadas de déficits de aprendizagem e de memória em um teste de evitação passiva, e de uma diminuição da latência de retirada da cauda em um teste de cauda no final da semana 6. Os resultados também mostraram que o tratamento crônico com carvacrol a uma dose de 25 mg / kg aliviou os déficits de memória, sem efeito sobre rotações e hiperalgesia em ratos lesados. Em conclusão, o carvacrol melhora a deficiência de memória em ratos com DP e, portanto, pode servir como uma terapia complementar para aliviar os déficits de memória em pacientes com DP.

Palavras-chave: Carvacrol; 6-hidroxidopamina; memória; atividade motora; hiperalgesia; doença de Parkinson.

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 bradykinesia¹. In addition to motor dysfunctions, cognitive deficits such as learning and memory impairments and dementia are seen in a high percentage of PD patients². The proportion of PD patients with dementia is 25–30%, up to six times higher than in healthy people³. Moreover, PD patients suffer from painful sensations that have been described as five different types: musculoskeletal pain (due to parkinsonian rigidity, rheumatological disease or skeletal deformity), radicular-neuropathic pain (due to a root lesion, focal or

peripheral neuropathy), dystonic pain (related to antiparkinsonian medication), central neuropathic pain (related to antiparkinsonian medication) and akathisia (during off-periods or drug induced)⁴.

Pharmacotherapy with L-DOPA and DOPA-decarboxylase inhibitors is still the most effective treatment for motor symptoms, but this type of therapy has no effect on cognitive deficits in PD^{5,6}. Considering the clear impact of cognitive deficits on the quality of the PD patient's life, it is valuable to investigate the treatments affecting non-motor symptoms, such as cognitive deficits and pain, in animal models of PD.

Carvacrol (CAR, 2-methyl-5-isopropylphenol) is a phenolic monoterpene abundantly present in the essential oil of the

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Conflict of interest: There is no conflict of interest to declare.

Received 09 May 2017; Received in final form 29 October 2017; Accepted 08 November 2017.

Support: This study was supported by Isfahan University of Medical Sciences.



family lamiaceae. Carvacrol has been reported to have many pharmacological benefits, including antibacterial, antifungal, antioxidant, antinociceptive, anti-inflammatory, anti-apoptosis and anti-cancer activities⁷. Carvacrol also exerts several actions on the neuronal system including acetylcholinesterase inhibition^{8,9}, as well as having anxiolytic¹⁰ and antidepressant¹¹ properties. It also modulates central neurotransmitter pathways, such as dopaminergic, serotonergic and GABAergic systems⁷.

Based on the antioxidant activity of carvacrol and its effects on cholinergic and dopaminergic systems, we decided to examine whether carvacrol could improve the motor and memory deficits and pain in the 6-OHDA model of Parkinson's disease.

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 ($22 \pm 2^\circ\text{C}$) 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 in accordance with 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 to five groups, as follows:

Group 1: sham-operated group (injection of 0.2% ascorbate-saline into the left medial forebrain bundle (MFB), 1% Tween 80 ip, $n = 7$);

Group 2: lesioned group (16 μg 6-OHDA into the MFB, 1% Tween 80 ip, $n = 7$);

Group 3: Carvacrol-treated lesioned group (16 μg 6-OHDA into the MFB, 25 mg/kg carvacrol ip, $n = 10$);

Group 4: Carvacrol-treated lesioned group (16 μg 6-OHDA into the MFB, 50 mg/kg carvacrol ip, $n = 10$);

Group 5: Carvacrol-treated lesioned group (16 μg 6-OHDA into the MFB, 100 mg/kg carvacrol ip, $n = 7$).

Carvacrol was emulsified with 1% Tween 80 (Sigma, USA) and dissolved in normal saline. The animals were treated with carvacrol at doses of 25, 50 and 100 mg/kg, intraperitoneally, one week before the surgery until six weeks after surgery. The sham-operated group received 1% Tween 80 dissolved in normal saline at the same volume as the treated groups.

Surgery and 6-OHDA lesion

Under chloral hydrate (450 mg/kg, ip) anesthesia, 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 (16 μg /4 μl 0.2% ascorbate-saline) 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 an identical volume of the ascorbate-saline as the vehicle. The injection rate was 1 $\mu\text{l}/\text{min}$ 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 cage and kept warm until recovery from anesthesia was complete.

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. The number of ipsilateral rotations was counted as positive scores and those of contralateral rotations as negative scores. The net number of rotations were defined as the difference between the rotations in both directions¹³.

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 time¹⁴.

Tail-flick test

The animal's response to phasic pain was tested by measuring the latency of tail flick to a high intensity light beam. The test was performed with a Tail Flick instrument that gives an automatic recording of tail-flick latency to radiant heat. The animal was placed on the recording platform of the apparatus where it was kept under painless restraint, with its tail placed on the radiant heat window. When the animal flicked its tail, a photocell was activated and the time between activation of the heat source and tail flick latency was recorded. For each animal, the thermal stimulus was applied on three different parts of the tail and the latency was considered as the mean of three measurements¹⁵. A cut-off exposure time of 15 seconds was set to prevent tissue damage.

Dissection and homogenization

After completion of the behavioral testing, the animals were euthanized and the brains were removed from the skulls on day 42. The striatum was dissected out and weighed. A 10% (w/v) tissue homogenate was prepared in NaCl solution.

Lipid peroxidation levels

The lipid peroxidation level of the 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 were 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 nm¹⁶. The level of TBARS was calculated by: $C (M) = \text{absorbance} / 1.65 \times 10^5$.

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 μ l homogenate and the sample absorbance was read at 412 nm against the tris-EDTA buffer alone (A1). Then, 20 μ l 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: $(A2-A1-B) \times 1.07 / 0.05 \times 13.6^{16}$.

Histology

The brains were removed and stored in 10% formalin for 72 hours. The brains were sectioned coronally at 40 μ m 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 (Figure 1) was determined by reference to a rat brain atlas¹².

Statistical analysis

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

RESULTS

Effects of carvacrol on rotational behavior

The results showed that apomorphine hydrochloride administration (2 mg/kg, ip) produced contralateral rotations towards the lesion side in 6-OHDA-lesioned rats at the end of the 2nd (Figure 2A) and 6th week (Figure 2B),

indicating unilateral damage to the left MFB. No such rotations were observed in the sham group rats.

Analyzing data with one-way ANOVA revealed a significant difference in rotations between groups ($p < 0.001$) at weeks 2 and 6. Further analysis with Tukey's *post hoc* test showed that the number of contralateral rotations in the 6-OHDA-lesioned group and the carvacrol-treated lesioned groups were significantly increased compared to the sham group ($p < 0.001$) at weeks 2 and 6, but there was no difference between the treated and not-treated groups (Figures 2A and 2B).

Effects of carvacrol on passive avoidance memory

As shown in Figure 3, the step-through latency of 6-OHDA-lesioned rats (128.8 ± 47.38) was shorter than the sham group rats (293 ± 7) at the end of week 6 ($p < 0.05$, Figure 3). Moreover, treatment of lesioned rats with carvacrol at a dose of 25 mg/kg significantly increased the latency (281 ± 19) compared with lesioned rats (128.8 ± 47.38) ($p < 0.05$).

Effects of carvacrol on tail flick latency

According to the results, there was a significant decrease in tail flick latency in 6-OHDA-lesioned rats compared to sham group rats at week 6 ($p < 0.05$, Figure 4). No significant difference in latency to tail flick was observed following carvacrol administration in the 6-OHDA-lesioned groups (Figure 4).

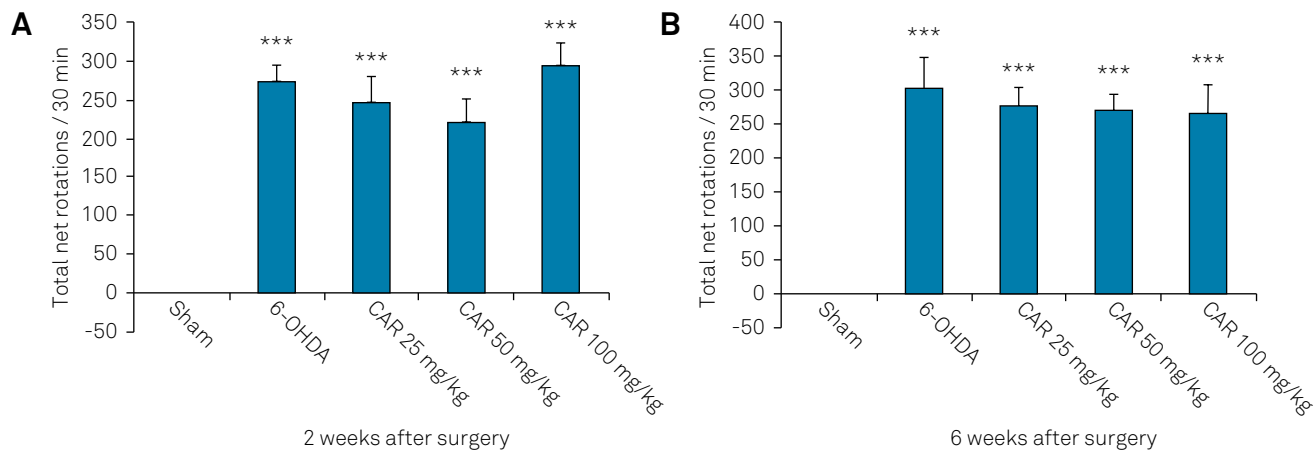
Effects of carvacrol on lipid peroxidation levels

Injection of 6-OHDA into the MFB resulted in significant elevation of TBARS level in the striatum ($p < 0.05$). In addition, the results showed that treatment of lesioned rats with carvacrol at doses of 25, 50 and 100 mg/kg for six weeks did not change the increased TBARS level in the striatum (Figure 5).



mt: mammillothalamic tract, F: fornix.

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



***p < 0.001 vs sham group.

Figure 2. Apomorphine-induced rotations in the sham, 6-OHDA-lesioned group and carvacrol-treated lesioned groups at the end of the 2nd (2A) and 6th (2B) week after surgery. Carvacrol was administered ip daily at doses of 25, 50 and 100 mg/kg.

Effects of carvacrol on total thiol concentration

Total thiol concentrations in the striatum were significantly decreased in the 6-OHDA-lesioned animals compared to the sham group ($p < 0.05$). Treatment of lesioned rats with carvacrol at doses of 25, 50 and 100 mg/kg for six weeks did not change the decreased total thiol concentrations in the striatum (Figure 6).

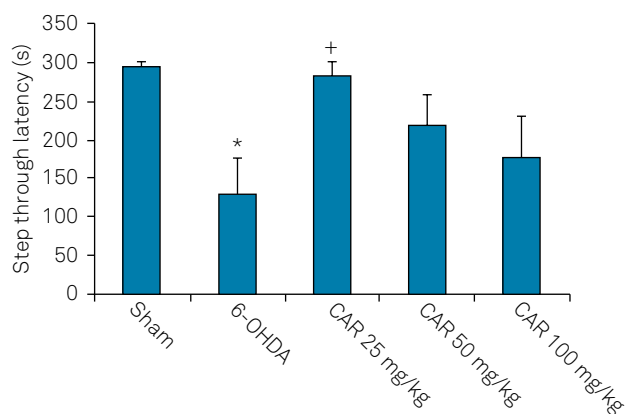
DISCUSSION

It has been demonstrated that 6-OHDA causes the degeneration of nigrostriatal dopaminergic neurons and produces motor and non-motor impairments such as cognitive deficits similar to those observed in patients with PD^{14,17,18}. Therefore, it was used as a valid model of PD. Our results also confirmed these changes. The administration of

6-OHDA into the MFB produced motor and memory deficits and reduced the pain threshold.

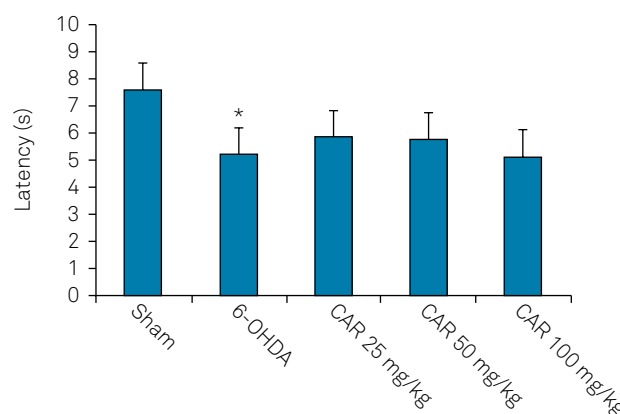
The 6-OHDA compound is a dopaminergic neurotoxin that undergoes auto-oxidation and produces cytotoxic hydrogen peroxide, reactive oxygen species and catecholamine quinones, which attack intracellular nucleophilic groups¹⁹. The increase in reactive oxygen species levels causes abnormalities in cell structure and metabolism and eventually leads to neuronal degeneration²⁰. In line with this, our results showed that microinjection of 6-OHDA into the MFB caused oxidative damage to the membrane, as evidenced by increased levels of TBARS and decreased total thiol concentration in the striatum at the end of week 6.

In the present study, the microinjection of 6-OHDA into the left MFB resulted in motor deficits that were observed by increased rotations. The unilateral lesion of the nigrostriatal



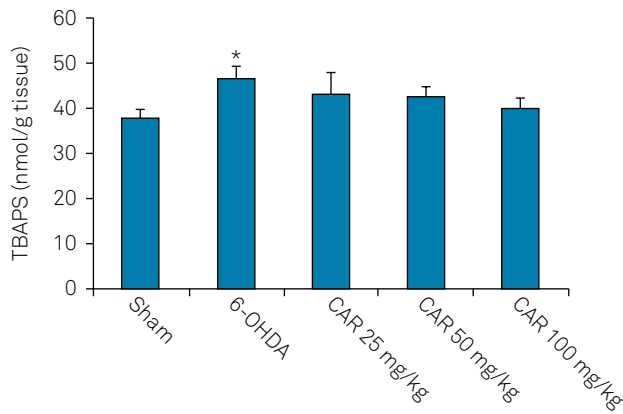
*p < 0.05 vs sham group, +p < 0.05 vs 6-OHDA-lesioned group.

Figure 3. Step-through latencies in the passive avoidance test in the sham, 6-OHDA-lesioned rats and lesioned rats treated with carvacrol at doses of 25, 50 and 100 mg/kg at the end of week 6. Data are mean \pm SEM.



*p < 0.05 vs sham group.

Figure 4. Tail flick latencies in the sham, 6-OHDA-lesioned rats and lesioned rats treated with carvacrol at doses of 25, 50 and 100 mg/kg at the end of week 6. Data are mean \pm SEM.

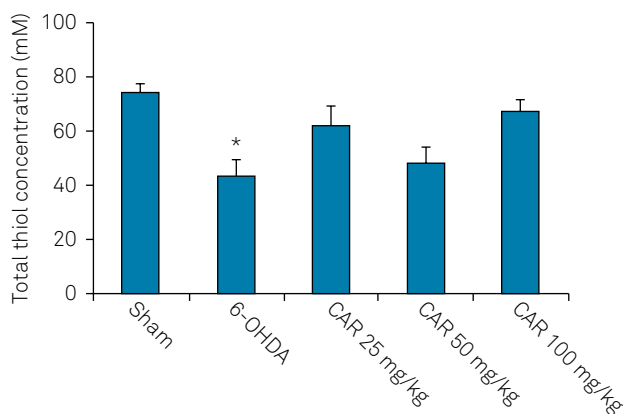


*p < 0.05 vs sham group.

Figure 5. Lipid peroxidation levels in the striatum of the sham, 6-OHDA-lesioned rats and lesioned rats treated with carvacrol at doses of 25, 50 and 100 mg/kg at the end of week 6. Data are mean \pm SEM.

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 apomorphine²¹. Apomorphine-induced rotations in 6-OHDA-lesioned rats is a reliable marker for the nigrostriatal dopamine depletion. The symptoms of PD appear when about 60–80% of the dopamine levels and 50–60% of dopaminergic neurons in the substantia nigra are lost²².

The present study also examined the potential therapeutic effect of carvacrol, as an antioxidant agent, in a 6-OHDA model of PD. Our results showed that treatment with carvacrol at doses of 25, 50 and 100 mg/kg did not decrease the apomorphine-induced rotations in rats. This is in contrast to prior studies, which report the protective effects of carvacrol on oxidative insults such as cerebral ischemia-reperfusion²³. The reasons for this discrepancy could be related to the



*p < 0.05 vs sham group.

Figure 6. Total thiol concentrations in the striatum of the sham, 6-OHDA-lesioned rats and lesioned rats treated with carvacrol at doses of 25, 50 and 100 mg/kg at the end of week 6. Data are mean \pm SEM.

dosage and duration of treatment. The initial oxidative stress produced by 6-OHDA might be attenuated by the antioxidant activity of carvacrol; however, when there is substantial ongoing oxidative stress and neurodegeneration²⁴ – as in the 6-OHDA model of PD – the antioxidant response wanes or is overwhelmed over time and, at that point, carvacrol cannot act as an antioxidant.

Furthermore, our results also showed that unilateral lesion to the left MFB significantly reduced the pain sensation threshold in the tail flick test. This is consistent with previous findings, which have reported that bilateral 6-OHDA lesions decreased the latency of the hind paw lick in the hot plate test²⁵ and enhanced sensitivity to a wide range of thermal and mechanical painful stimuli²⁶. The precise mechanisms underlying alteration in the withdrawal response to thermal and mechanical stimulation in the 6-OHDA rats is unclear. Neurophysiological, clinical and behavioral experiments indicate that, other than motor control, the basal ganglia are also involved in pain processing²⁷. Experimental studies have shown that striatal dopamine is important for modulating nociceptive behavioral responses²⁷, an effect that appears mediated through dopamine D2 receptors²⁵. The striatum has direct and indirect efferent connections to various brainstem structures involved in the descending pain modulation system²⁷. It has been suggested that the upregulation of dopamine D2 receptors in the striatum in the 6-OHDA rats may lead to a decrease in the activity of the descending pain modulation system.

In the present study, the unilateral 6-OHDA lesioned rats were treated with carvacrol to evaluate its possible effect on pain sensation in an animal model of PD. The results showed that treatment with carvacrol did not change the pain sensation threshold in the tail flick test in PD animals. This could also be attributed to the lack of antioxidant activity of carvacrol in this study.

Moreover, cognitive decline is a main non-motor symptom of PD that predisposes the majority of patients to progression into dementia²⁸. Parkinson's disease patients complain of executive dysfunction and deficiencies of working memory that resemble those created by frontal lobe injury²⁹. The 6-OHDA-induced neuronal loss in the substantia nigra has also been shown to result in learning and memory impairments^{14,30}. In line with previous studies, our results also showed that short-term memory in the step-down avoidance task had deteriorated in the rats with 6-OHDA-induced PD, and treatment with carvacrol at a dose of 25 mg/kg improved short-term memory.

Cognitive deficits in PD patients might result from non-dopaminergic dysfunction, as degeneration of noradrenergic, serotonergic and, most importantly, cholinergic systems have been reported in PD³¹. Significant loss of cholinergic forebrain neurons has been reported in PD-affected brains³². It also has been reported that the loss of cholinergic cells in the nucleus basalis of Meynert (where Lewy bodies are frequently found)

is greater than that seen in Alzheimer's disease³³. The nucleus basalis of Meynert is the main source of cholinergic projections to the cerebral cortex and degenerates in PD. In addition, choline acetyltransferase activity has been found to be significantly decreased in the cortex of parkinsonian subjects³⁴.

Our findings indicated that carvacrol ameliorated short-term memory impairment in rats with PD. It has also been shown that carvacrol improved spatial memory impairments induced by scopolamine, a muscarinic receptor antagonist, in rats³⁵. More importantly, the acetylcholinesterase inhibitory activity of carvacrol has been shown in several studies^{8,9}. Therefore, the effect of carvacrol on memory improvement could be due to its anticholinesterase activity

and modulation of the cholinergic system. In agreement with this, it has been reported that acetylcholinesterase inhibitors have a beneficial effect on cognitive performance in PD³⁶.

In conclusion, the present study demonstrated that carvacrol improves short-term memory impairment in rats with PD. Considering that L-DOPA therapy for PD just relieves motor symptoms, we suggest that carvacrol may serve as an adjunct therapy for the alleviation of memory deficits in patients with PD.

Acknowledgements

The results presented in this work have been taken from a student's thesis. This study was supported by Isfahan University of Medical Sciences.

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