

6-Hydroxydopamine-Induced Neurotoxicity in Rat Model of Parkinson's Disease: Is Reversed via Anti-Oxidative Activities of Curcumin and Aerobic Exercise Therapy

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Summary

In the rat model, 6-hydroxydopamine (6-OHDA) known as a selective catecholaminergic neurotoxin used chiefly in modeling Parkinson's disease (PD). Continuous aerobic exercise and curcumin supplementations could play a vital role in neuroprotection. This study aimed to explore the neuroprotective roles of regular aerobic exercise and curcumin during PD. For this, rats were treated as follows for 8 consecutive weeks (5 d in a week): For this, animals were orally treated with curcumin (50 ml/kg) alone or in combination with aerobic exercise. Compared with a control group, induction of PD by 6-OHDA increased the amount of α -synuclein protein and malondialdehyde levels and decreased the number of substantia nigra neurons, total antioxidant capacity, and glutathione peroxidase activity in brain tissue. All these changes were abolished by the administration of curcumin with aerobic exercise treatments. Activity behavioral tests also confirmed the above-mentioned results by increasing the rod test time and the number of rotations due to apomorphine injection. Histopathology assays mimic the antioxidant activity and behavioral observations. Combined curcumin with aerobic exercise treatments is potentially an effective strategy for modifying the dopaminergic neuron dysfunction in 6-OHDA-induced rats modeling PD via dual inhibiting oxidative stress indices and regulating behavioral tasks.

Key words

6-OHDA • Parkinson's disease • Aerobic exercise • Curcumin • Behavioral tasks • Oxidative stress

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Introduction

The health science has faced with main healthcare and societal challenge for handling of neurodegenerative diseases, and common therapeutic methods often results in unsatisfactory outcomes. Meanwhile, there is an increasing interest in topic of combined aerobic training with natural antioxidant therapy, due to the overall brain damages increase in high-income countries, but there is still much to know concerning the effect of toxic substances on the central nervous system.

After Alzheimer's disease, the second most common neurodegenerative disorder is Parkinson's disease (PD) which can affect every aspect of a person's life [1]. Generally, the pathological symptoms of PD include the loss of dopaminergic neurons in the dense black matter (SNpc) and the accumulation of α -synuclein-containing Lewy bodies (LBs) in the cytoplasm that affects 1-2 per 1000 of the population at any time [2]. These are the cells that manufacture the molecule dopamine; a chemical messenger that transmits signals between two regions of the brain to coordinate activity and helps control muscle movement [3].

Several genetic risk factors have now been characterized, as well as several genes which cause rare familial forms of PD. Approximately 5-10 % of PD cases have different genetic causes [4]. However, a full understanding of the mechanism by which these genes function is still lacking. One of the genes involved in

heredity is the α -synuclein or SNCA gene. This gene is the dominant gene and the main component of LBs, which has been well-validated as susceptibility genes for PD [5]. Available data suggest that α -synuclein mediates dopamine secretion, synthesis, or storage regulation [6]. Thus, defects in dopamine have been implicated in the pathogenesis of PD.

In laboratory animal model, 6-hydroxydopamine (6-OHDA) known as a selective catecholaminergic neurotoxin used chiefly in modeling PD [5]. Depletes brain catecholamine levels via uptake and accumulation by a transport mechanism specific to these neurons [7]. In consideration of the major pathogenic pathways that 6-OHDA influence, previous study revealed that it involved in neuronal loss and behavioral alterations characteristic for parkinsonism [8].

Natural compounds can be used to treat neurodegenerative diseases. Curcumin is a polyphenolic compound (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) derived from turmeric (*Curcumin longa* L.). It has been reported that curcumin plays an important role in the modulation of oxidative stress [9], and inflammation [10]. Curcumin has been known to have some physiological and pharmacological properties such as anti-inflammatory, antioxidant, hypoglycaemic, and antihyperlipidemic activities [11, 12]. Protective effects of curcumin on neuroprotective have been reported [13]. Improvements in neurological disease, epilepsy, cerebral ischemia, and depression, as well as brain damage, have been demonstrated by curcumin [14]. Curcumin, due to its limited solubility in aqueous solvents (11 mg/ml in aqueous buffer with pH 5) and low permeability in intestinal epithelial cells, is ranked IV in the BCS drug classification. Poor solubility and bioavailability of curcumin significantly affect its therapeutic application [15].

Nowadays, assessment of aerobic exercise activities during neurodegenerative disorders is a significant issue owing to an increased ecotoxicological data looking at the impact of physical activities' side effects on neuroprotective roles. Aerobic exercise increases the brain's antioxidant capacity [16]. The effect of the treadmill on increasing the capacity of endogenous neurogenesis in PD in rats has been studied and results showed this strategy increased cell proliferation and migration of nerve stem cells to the lesion site [17]. Thus, physical exercise for improving adult neurogenesis could know as a good strategy to prevent cognitive decline in neurodegenerative diseases.

Based on our knowledge until now no studies are available that examine the effect of combined curcumin with aerobic exercise treatments on neurotoxicity 6-OHDA-treated in rat modeling PD. Thus, our investigation tested the hypothesis that alone or combined curcumin with endurance activities protects brain-injured 6-OHDA toxicity via dual inhibiting oxidative stress indices and regulating behavioral tasks. The results provide a novel mechanistic approach concerning 6-OHDA-induced neurotoxicity.

Material and Methods

Chemicals

Unless otherwise indicated, all reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). The kits to evaluate oxidative stress indices levels were purchased from Nanjing Jiancheng Bioengineering Institute (China), except for glutathione peroxidase (GPx) (Biodiagnostic, Egypt).

Animals and Ethics

Animals obtained from the Pasteur Institute of Iran (Karaj, Iran) were housed in a temperature and light-controlled conditions. Bodyweight and food and water consumption per animal were recorded weekly. No significant differences were found in these measures between animals in any of the experimental groups during the experiment. The study protocol was carried out in compliance with the guidelines for maintenance and handling of laboratory animals and techniques approved by the Ethics Review Committee of Tabriz University of Medical Sciences, Iran (approval no.: 03524.1.45/25).

Treatments

36 male adult Sprague Dawley rats, an average 210.25 g and 8 week old, were challenged as follows (n=6/group) for 8 consecutive weeks (5 d in a week): normal control (NC); surgical sham (SH); patient control (PD; intrastriatal single-dose injection of 12.5 μ g of 6-OHDA into the left striatum; Lot: STBH3207V; [18]); positive control (PC; Levodopa 12 mg/kg b.wt. twice per day by gavage for 21 d after the onset of the PD); curcumin (CT; 50 ml/kg b.wt. per day); curcumin+aerobic exercise (CET; 30 min/d and 5 d/week for 8 weeks with speed 11 m/min; [19]). The method of Roghani *et al.* [18] was used to establish the Parkinson's model in the PD groups, PC, CT, CET.

Table 1. The ingredients of lysis buffer for western blotting

Tris-HCL	EDTA	NaCl	Sodium Deoxycholate	SDS	Protease inhibitor cocktail	Triton [NP40 (1 %)]
500 μ l, pH=8	0.003 g	0.08 g	0.025 g	0.01 g	1 Tablet	10 μ l

Sampling

Rats were selected that did not have staggering and rotating behavior (net rotation less than four times per minute) after injection of apomorphine hydrochloride (Sigma, Germany, Batch: SLBF5500V). First, the animals were anesthetized with ketamine (Bremer, Germany) (intraperitoneal, 100 mg/kg b.wt.) and xylazine (Alfasan, Holland) (intraperitoneal, 10 mg/kg b.wt.). The rats head was then fixed symmetrically inside the stereotaxic device (incisor bar -3.3 mm), and the surface of the skull was cleaned. Injection was performed based on the coordinates obtained from Atlas Paxinos (AP=-5.5mm, DV=-7.3 mm, ML=-2.6 mm).

Behavioral tests

At the end of the treatment period, behavioral tests of rotation with apomorphine [18], rod test [20], and spatial memory test with the Morris water maze device [21] were performed.

Determination of oxidative index

The brain and blood of some other rats were obtained after decapitating. The rat's brain was quickly transferred to a freezer at -80 °C for freezing. The samples were then homogenized in a saline phosphate buffer solution in a ratio of 1 to 10 and centrifuged at 12000 rpm for 15 min at 40 °C. The above solution was used to measure MDA (malondialdehyde) and GP_x kits [22]. Rats' blood serum was used to measure TAC serum (total antioxidant capacity; [23]) (produced by Rendox Company).

Histopathological studies

After 21 and 28 d, the brains were fixed in Bouin's solution for 24 h, the brains were dehydrated in a series of graded ethanol, and then embedded in paraffin, Then were cut into 8 μ m sections thickness using the microtome (LEICA RM2145) and stained with 0.05 % chrysalis violet based on the manufacturer's instructions, and the number of dopamine neurons stained in the substantia nigra was counted. The optical microscope was used to observe the histopathological changes (Olympus, Tokyo, Japan). 10 visual fields per slide and six sections

per group were selected randomly for analysis under 40 \times magnifications.

Western blotting

100 mg of brain tissue was taken, ground to a powder with liquid nitrogen, and homogenized in lysis buffer (Table 1). After standing at 4 °C for 20 min of homogenization, the solution was centrifuged at 12000 rpm/min at 4 °C for 10 min. Afterward, the resultant supernatants were collected and used for the next step. Protein concentrations of samples were determined at λ =630 nm using a BCA kit (Beyotime Biotechnology, Shanghai, China).

In brief, 50 μ g brain protein was added onto 10 % SDS polyacrylamide electrophoresis gels (into the running buffer). Afterward, the injected proteins with different molecular mass and charge were separated according to isoelectric points. Then, the electrophoretically separated components were transferred from the gel to the NC membrane. The NC membrane was placed face-up in 5 % skim milk powder for blocking and incubated at 4 °C overnight with a primary antibody diluted to the appropriate concentration followed by incubation with a secondary antibody for 2 h. Primary antibodies include: Mouse anti-Beta Actin monoclonal antibody (1:1500), rabbit anti-TGM2 polyclonal antibody (1:500), rabbit anti-Anxa5 polyclonal antibody (1:1000), rabbit anti-PI3K polyclonal antibody (1:1000), rabbit anti-p-PI3K (p85) monoclonal antibody (1:800), rabbit anti-p-AKT (Phospho-T308) monoclonal antibody (1:1000), rabbit anti-Foxo1 polyclonal antibody (1:800), rabbit anti-Bcl-2 polyclonal antibody (1:1000) and rabbit anti-Caspase 3 polyclonal antibody (1:1000) were purchased from Proteintech Group (Wuhan, China); while, rabbit anti-AKT1 antibody (1:1500) was purchased from Bioss (Beijing, China). Then, the Electro-Chemi-Luminescence reagent was evenly coated on the NC membrane for 2 min. Images were collected and the target band was analyzed by optical density by Fluor Chem Q System (Alpha Innotech, CA, USA). All blots were repeated at least three times.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, USA). The results were expressed as mean±standard deviation (SD) and performed with one-way analysis of variance (ANOVA) followed by Tukey post hoc tests and values of $p < 0.05$ were considered as statistically significant.

Results

Effects of orally administrated curcumin single

or combined with aerobic exercise on antioxidant activity and α -synuclein protein levels in 6-OHDA-induced rats modeling PD are presented in Figure 1 and Table 2, receptively.

Considering the vehicle group, curcumin-treated animals showed significantly lower α -synuclein protein and MDA concentrations in brain tissue, while ameliorating TAC and GP_x levels in blood serum and brain tissue, receptively. Meanwhile, a combination of curcumin and aerobic exercise produced the best therapeutic effect, resulting in a reduction of β -synuclein protein and MDA levels, plus a considerable

Table 2. Effects of orally administrated of curcumin single or combined with aerobic exercise on antioxidant activity and α -synuclein protein levels in 6-OHDA-induced rats modeling Parkinson's disease

Groups	Serum TAC	Brain GPx	Brain MDA	Brain α -synucleine / β -Actin
NC	0.43±0.02 ^c	5.05±0.02 ^c	0.59±0.01 ^c	0.23±0.005 ^d
SH	0.41±0.01 ^c	5.01±0.01 ^c	0.57±0.01 ^c	
PD	0.29±0.01 ^a	3.85±0.03 ^a	1.39±0.01 ^a	0.838±0.005 ^a
PC	0.33±0.01 ^b	4.41±0.02 ^b	1.28±0.02 ^b	0.403±0.004 ^c
CT	0.43±0.02 ^c	4.9±0.02 ^c	0.89±0.01 ^c	0.844±0.000 ^a
CET	0.41±0.02 ^c	4.65±0.03 ^d	0.98±0.03 ^d	0.637±0.000 ^b

Values are given as means ±S.D. of 6 rats. Superscripts (a-c) show significant differences in each column ($p < 0.05$). TAS (% of control): total antioxidant status; MDA (nmol/mg protein): malondialdehyde; GSH-Px (μ g/g tissue): glutathione peroxidase. Normal control (NC); surgical sham (SH); patient control (PD; intrastratial single-dose injection of 12.5 μ g of 6-OHDA into the left striatum); positive control (PC; Levodopa 12 mg/kg b.wt. twice per day by gavage for 21 d after the onset of the PD); curcumin (CT; 50 ml/kg b.wt. per day); curcumin+aerobic exercise (CET; 30 min/d and 5 d/week for 8 weeks with speed 11 m/min).

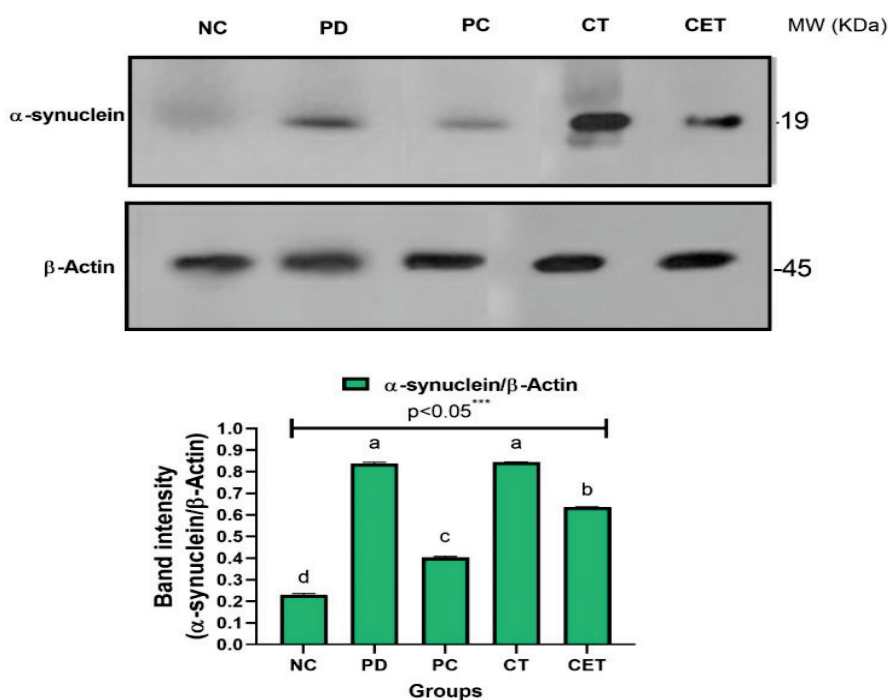


Fig. 1. Changes in the concentrations of α -synuclein protein in 6-OHDA-induced rats modeling Parkinson's disease. All data were expressed as relative values against their respective control group. The values are presented as mean ±SEM (n=6). β -actin was used as an internal control. (a-d) mean values with common letter (s) above bars do not differ significantly ($p < 0.05$). Normal control (NC); patient control (Parkinson's disease (PD) intrastratial single-dose injection of 12.5 μ g of 6-OHDA into the left striatum); positive control (PC; Levodopa 12 mg/kg b.wt. twice per day by gavage for 21 d after the onset of the PD); curcumin (CT; 50 ml/kg b.wt. per day); curcumin+aerobic exercise (CET; 30 min/d and 5 d/week for 8 weeks with speed 11 m/min).

improvement in blood serum TAC consideration.

There was no significant difference in the amount of TAC factor the between NC, SH, and CET groups, and its mean was higher in the PD group than in other groups. Also, the mean activity of the GPx enzyme in the CT group was significantly higher than in the PD, PC, and CET groups ($p < 0.05$), and in the CET group was lower than in the NC and CT groups. The mean MDA of brain tissue in the CT group was significantly lower than in the PD, PC, and CET groups ($p < 0.05$), and in the CET group was higher than NC and CT groups (Table 2).

The Table 3 shows behavioral tasks including number of induced rotations, duration of rod test, and spatial memory in each group. At the same time, activity behavioral tests confirmed the antioxidant activity results. administration of curcumin single or combined with aerobic exercise improved activity behavioral tests by increasing the rod test time and spatial memory observations.

Figure 2 (sections 1 until 5) show representative sections of brain tissue of rats in the no-6-OHDA and 6-OHDA groups. In no-6-OHDA groups the histopathological sections were normal (completely) in

the control group (sections 1). Meanwhile, the administrated of an intrastriatal single-dose injection of 12.5 μg of 6-OHDA into the left striatum caused damage to the number of dopaminergic neurons in the substantia nigra (section 2). In the Levodopa and combination groups, treatments enabled the repair of the brain tissue by increasing the number of dopaminergic neurons in the substantia nigra (sections 3, 4, and 5).

Discussion

The prevalence of neurodegenerative diseases is increasing in worldwide. Meanwhile, [central nervous system \(CNS\)](#) drugs causes a wide range of adverse effects on health, one being an increase in PD. Nowadays, assessments of antioxidant capacity in rodents are now a significant issue owing to the increase of ecotoxicological data looking at the impact of cytotoxicity drugs on the CNS.

In the present study, to produce animal models of PD used 6-OHDA as well as an increase in α -synuclein protein and MDA (lipid peroxidation index) was observed, as well as a decrease in the number of neurons

Table 3. Effects of orally administrated of curcumin single or combined with aerobic exercise on behavioral tasks (number of induced rotations and duration of rod test (Sec)) and spatial memory in 6-OHDA-induced rats modeling Parkinson's disease (mean \pm SD)

Groups	Test	NC	SH	PD	PC	CT	CET
1	Rotation	0.00 \pm 00	0.00 \pm 00	0.00 \pm 00	0.00 \pm 00	0.00 \pm 00	0.00 \pm 00
	Rod	0.00 \pm 00	0.00 \pm 00	0.00 \pm 00	0.00 \pm 00	0.00 \pm 00	0.00 \pm 00
7	Rotation	0.00 \pm 00	0.00 \pm 00	0.33 \pm 0.51	0.33 \pm 0.51	0.33 \pm 0.51	0.5 \pm 0.83
	Rod	0.00 \pm 00	0.00 \pm 00	0.00 \pm 00	0.00 \pm 00	0.00 \pm 00	0.00 \pm 00
14	Rotation	0.00 \pm 00 ^a	0.00 \pm 00 ^a	3.66 \pm 1.21 ^b	3.83 \pm 1.16 ^b	3.83 \pm 1.16 ^b	3.83 \pm 1.16 ^b
	Rod	0.00 \pm 00 ^a	0.00 \pm 00 ^a	3 \pm 00 ^a	3 \pm 00 ^a	3 \pm 00 ^a	3 \pm 00 ^a
21	Rotation	0.00 \pm 00 ^a	0.00 \pm 00 ^a	3.66 \pm 1.21 ^b	2 \pm 0.89 ^b	2.66 \pm 1.36 ^b	3.16 \pm 1.6 ^b
	Rod	0.00 \pm 00 ^a	0.00 \pm 00 ^a	3 \pm 00 ^a	2.41 \pm 0.58 ^{ab}	2.41 \pm 0.58 ^{ab}	2.08 \pm 0.49 ^b
28	Rotation	0.00 \pm 00 ^a	0.00 \pm 00 ^a	3.66 \pm 0.81 ^c	1 \pm 0.63 ^b	0.5 \pm 0.54 ^{ab}	0.33 \pm 0.51 ^{ab}
	Rod	0.00 \pm 00 ^a	0.00 \pm 00 ^a	3 \pm 00 ^c	1.66 \pm 0.75 ^b	1.75 \pm 0.52 ^b	0.33 \pm 0.4 ^a
Spatial memory	TTQ	11.8 \pm 0.05 ^e	11.847 \pm 0.05 ^e	7.66 \pm 0.14 ^a	9.89 \pm 0.06 ^b	9.65 \pm 0.09 ^c	11.44 \pm 0.05 ^d
	DT	123266 \pm 180 ^e	123535 \pm 152 ^e	5762 \pm 73 ^a	107469 \pm 1007 ^b	114817 \pm 2779 ^c	44111 \pm 2114 ^d

Normal control (NC); surgical sham (SH); patient control (Parkinson's disease (PD); intrastriatal single-dose injection of 12.5 μg of 6-OHDA into the left striatum); positive control (PC; Levodopa 12 mg/kg b.wt. twice per day by gavage for 21 d after the onset of the PD); curcumin (CT; 50 ml/kg b.wt. per day); curcumin+aerobic exercise (CET; 30 min/d and 5 d/week for 8 weeks with speed 11 m/min). DT: distance traveled (cm); TTQ: traveled time in the target quarter (sec). Superscripts (a-d) show significant differences in each row ($p < 0.05$).

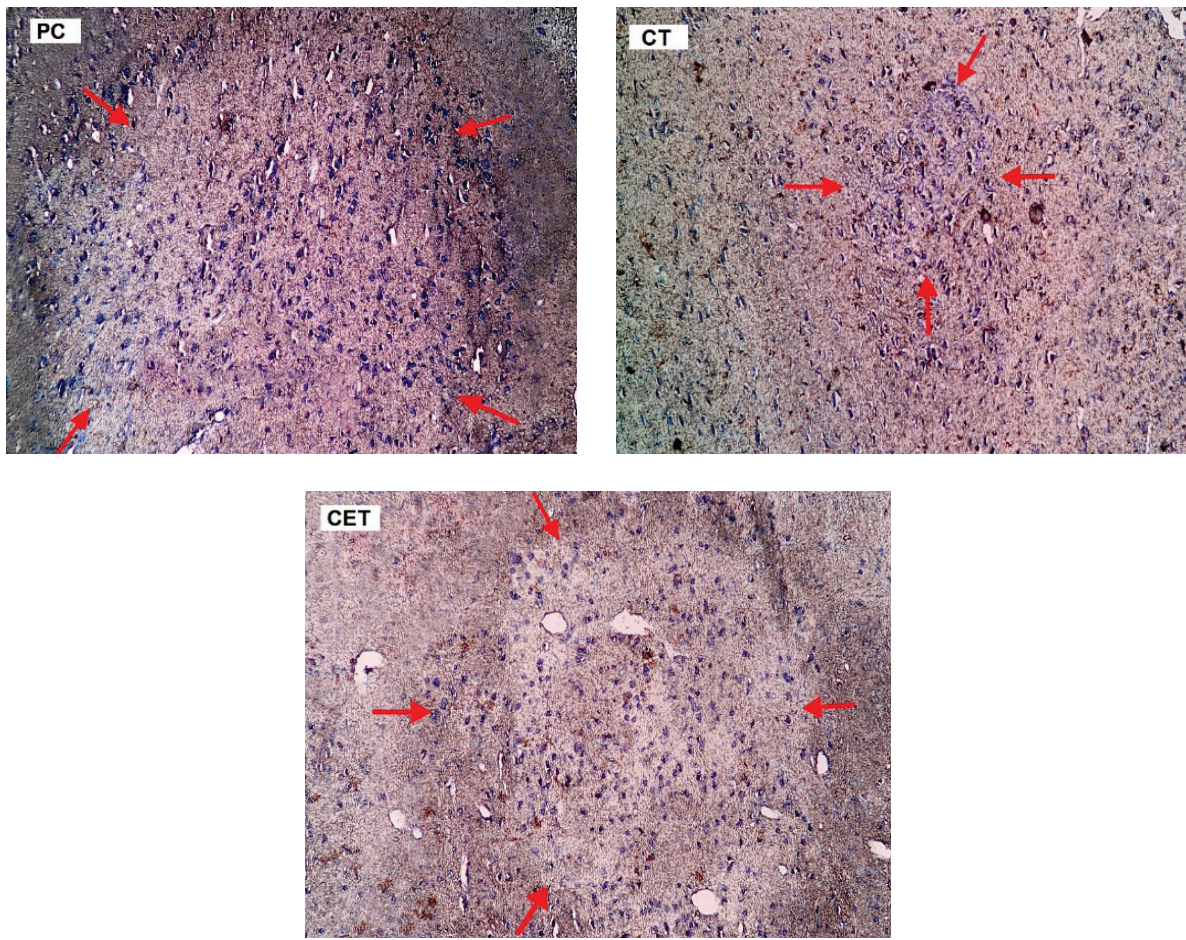


Fig. 2. Brain histopathological structure and the regeneration of dopaminergic neurons in the substantia nigra of rats modeling Parkinson's disease (PD) after 56 days simultaneously mentioned below treatments: normal control (NC); patient control (PD; intrastriatal single-dose injection of 12.5 μ g of 6-OHDA into the left striatum); positive control (PC; Levodopa 12 mg/kg b.wt. twice per day by gavage for 21 d after the onset of the PD); curcumin (CT; 50 ml/kg b.wt. per day); curcumin+aerobic exercise (CET; 30 min/d and 5 d/week for 8 weeks with speed 11 m/min).

in the substantia nigra by selectively destroying the nigrostriatal dopaminergic systems and a reduction in total antioxidant capacity, and a decrease in GPx activity in the brain. Lehmensiek *et al.* [24] suggested a mechanism of 6-OHDA-induced dopaminergic toxicity involving interaction of the mutant α -synucleins protein with the dopamine transporter molecule and subsequent acceleration of cellular energy depletion that might be relevant for the pathogenesis of PD. Our findings are well coordinated by the current literature on 6-OHDA toxicity, indicating that these cytotoxic drugs can cause nigrostriatal dopaminergic systems dysfunction. The reasons suggested for brain cell damage and decreased the number of neurons in the substantia nigra caused by 6 OHDA exposure in the current study maybe include the production of interaction with DNA and reactive oxygen species production.

In recent years, several investigations have been done to evaluate the possible therapeutic effects of

curcumin on PD by acting on various cellular pathways [25, 26, and 27]. The present study revealed that curcumin exposure decreased levels of α -synuclein protein and MDA, increased total antioxidant capacity, and GPx enzyme activity, by anti-oxidant and anti-inflammatory proprieties [27]. Curcumin indirectly plays an essential role in detoxifying oxidative stress and protecting against cell death by protecting the activity of antioxidant enzymes [28, 29]. There have been numerous reports on the modulatory effect of curcumin on lipid peroxidation and antioxidant enzymes following injuries such as hypoxia/ischemia and CNS injury [30, 31]. In parallel with previous studies, our study owed that curcumin inhibits the increase in the activity of antioxidant enzymes in the striatum induced by Parkinson's induction. There is ample evidence that curcumin may act as an inducer of autophagy and its function beyond purifying harmful protein aggregates. For example, curcumin induces neurogenesis by

activating autophagy [32].

Some studies have evaluated curcumin-based formulations to increase bioavailability, enhance the protective effects of curcumin, and investigate the anti-fibrillation effects and cytotoxicity of α -synuclein. For example, a nano-formulation containing curcumin silica nanoparticles prevents α -synuclein fibrillation and cytotoxicity. In neurons containing dopamine, a nano-formulation of curcumin prepared with lactoferrin protects against rotenone-induced neurotoxicity [33]. In the present study, the number of brain tissue neurons in the PD group was significantly lower than the NC, PC, and CET groups, and in the CET group was considerably higher than the PD and CT groups ($p < 0.05$). Curcumin reduces apoptosis in neurons by protecting them against Parkinson's symptoms in a rotenone-induced experimental model, according to histopathological findings (Fig. 2).

Thiol residues in GSH (a tripeptide) play an essential role in membrane protection. Lipid peroxidation and homeostatic disorders may be due to a significant reduction in this substance. Also, impairment of H_2O_2 decomposition and facilitation of OH formation due to decreased GSH content. This reduction can also cause mitochondrial damage by free radicals and subsequently selectively inhibit complex I activity [34]. Curcumin protection against glutathione has been shown in neurological diseases such as cerebral ischemia and Alzheimer's [35, 36]. In the present study, reversal of GSH induced by OHDA-6 was observed in mice in the curcumin group.

GPx, which plays a vital role in protecting membranes against toxicity, is supplied by GR. In the present study, the activity of these enzymes in the patient group was significantly reduced, and therefore the level of toxins increased and caused severe poisoning and cell death.

Aerobic exercise also improved the antioxidant capacity according to the pattern of curcumin effects in 6-OHDA-induced rats modeling Parkinson's. In Parkinson's patients, the physical exercise reduce

oxidative stress, increase mitochondrial biogenesis, autophagy, and antioxidant enzyme activity (superoxide dismutase), stimulation of neurotransmitters such as dopamine, and synthesis of nutritional factors could know as a possible application for the prevention or treatment of neurodegenerative disorders [37].

Aerobic exercise with curcumin leads to the best therapeutic effect. It significantly reduces the amount of α -synuclein protein in brain tissue, substantially improves serum's total antioxidant capacity, and reduces lipid peroxidation in brain tissue. It has been reported that activation of the cholinergic system has neuroprotective effects on dopaminergic neurons [38, 39, 40]. In agreement with the results of Nebrisi *et al.* study [38], combined curcumin with aerobic exercise treatments (single or combined) may offer a potential therapeutic approach to activation of the cholinergic system in PD. The results provide a novel mechanistic approach to 6-OHDA-induced neurotoxicity.

Conclusion

In summary, the data presented here demonstrated that natural compound (curcumin) with physical exercise (aerobic) (single or combined), are potentially an effective strategy for the improvement of PD disorder in rat model via dual inhibiting oxidative stress indices and regulating behavioral tasks, however, a full understanding of the mechanism by which these drugs function is still lacking.

Conflict of Interest

There is no conflict of interest.

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