

Original article

Toxic influence of chronic oral administration of paraquat on nigrostriatal dopaminergic neurons in C57BL/6 mice

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Keywords: *Parkinson's disease; paraquat; oxidative stress; tyrosine hydroxylase; dopamine transporter*

Background Paraquat (PQ; 1,1'-dimethyl-4,4'-bipyridinium), a widely used herbicide, has been repeatedly suggested as a potential etiologic factor for the development of Parkinson's disease (PD), owing to its structural similarity to the known dopaminergic neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). This study aimed to observe the influence of paraquat on nigrostriatal dopaminergic neurons in C57BL/6 mice.

Methods A total of 24 male C57BL/6 mice were assigned randomly to 3 groups: control group (treated by saline), PQ treated group, and MPTP treated group. Mice in PQ treated group were taken orally with PQ (10 mg/kg) daily for four months. Locomotor activity was measured. Level of dopamine (DA) and its metabolites levels in the striatum were measured by high-performance liquid chromatography with an electrochemical detector (HPLC-ECD), and tyrosine hydroxylase (TH) positive neurons were detected by using immunohistochemistry. At the same time, the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX), and the content of malondialdehyde (MDA) in substantia nigra were measured by spectrophotometry. mRNA expression of dopamine transporter (DAT) in dopaminergic neurons of substantia nigra was also determined by reverse transcription (RT)-PCR technique.

Results Locomotor activities were significantly impaired in the PQ treated group. Level of DA and its metabolites levels in the striatum were declined. The activities of SOD and GSH-PX were decreased, and the content of MDA was increased in PQ treated mice compared with that in control group. Numbers of TH positive neurons and the mRNA expression of DAT in substantia nigra of mice were also decreased after PQ taken orally for four months.

Conclusions The present study suggests that chronic oral administration of PQ could trigger dopaminergic neuron degeneration. Oxidative stress could be involved in the pathogenic mechanism of PD induced by PQ.

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Parkinson's disease (PD) is one of the most common neurodegenerative movement disorders. Its etiology or pathogenesis is unknown. The most marked pathological change in this disease is selective degeneration of the nigrostriatal dopaminergic neurons in the central nervous system. Considerable evidence suggests that exposure to environmental herbicides is associated with an increased risk for the development of Parkinson's disease.¹⁻³ Recently, the potential proparkinsonian role of paraquat has been discussed. Paraquat (PQ; 1,1'-dimethyl-4,4'-bipyridinium dichloride), a widely used herbicide, has been repeatedly suggested as a potential etiologic factor for the development of PD, owing to its structural similarity to MPP⁺ (1-methyl-4-phenylpyridinium ion), the active component of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP),^{4,5} a synthetic heroin compound which can induce Parkinson's syndrome.^{6,7} Several *in vivo* studies in rats and mice have shown that acute PQ administration may elicit a distinct decrease in the level of dopamine and the activity of spontaneous motor in mice.⁸⁻¹⁰ However, equivocal results have been reported by studies investigating the effect of exposure to PQ on selective nigrostriatal degeneration in young rodents.^{11,12}

It has been shown that oxidative stress may play a role in etiology of PD. Neuronal death occurs not only due to the

loss of energy, but also due to the damage resulting from free radicals.^{13,14} Dopamine transporters (DATs) are expressed in dopaminergic neurons and their expression may be a significant marker of dopaminergic nerve cells. It is known that DAT in nigrostriatal system maybe take part in the pathogenic mechanism of PD. DAT mRNA were decreased markedly in brain of animal model induced by MPTP and PD patients.^{15,16}

The present study was undertaken to investigate the influence of long-term chronic oral administration of PQ on nigrostriatal dopaminergic system of mice. Changes of oxidative stress and DAT expression in nigrostriatal system were also observed.

METHODS

Animal grouping and treatment

A total of 24 male C57BL/6 mice (8 weeks old; (20.20±1.97) g) were assigned randomly to three groups

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($n=8$ each): (1) control group: mice fed with saline; (2) PQ treated group: mice fed with PQ (Sigma, USA; 10 mg/kg daily) for four months; (3) MPTP treated group: mice fed with MPTP (Sigma; 20 mg/kg daily) for four months. Mice in each group were fed normally, using a transferpettor (20 μ l Socorex Swiss) to administer PQ. During the period of feeding, we observed the behavior changes of mice daily.

Behavioral testing

Spontaneous locomotor activity was measured using a computerized locomotion detection system equipped with photosensors (Scanet SV-10, MATYS, Toyama, Japan) as previously described.¹⁷ After taken orally with PQ for four months, mice were individually placed in a transparent cage (25 cm $\times$ 48 cm $\times$ 18 cm) and photobeam breaks were recorded every 2 minutes for 30 minutes to assess horizontal and vertical movements.

Analysis of dopamine and its metabolites

The two striatum were rapidly isolated from the cerebrum on an ice-cold glass plate and immediately frozen in liquid nitrogen. Samples were immediately weighed and homogenized in 1 ml ice-cooled 0.1 mol/L perchloric acid solution containing 0.2 μ g/ml L-isoproterenol hydrogen and 0.1 mmol/L EDTA with homogenizer. Tissue homogenates were centrifuged at 15 000 $\times g$ for 30 minutes at 4°C, and the supernatant was filtered and stored at -80°C until assay. Dopamine (DA), 3,4-dihydroxyphenyl acetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxytryptamine (5-HT), and its metabolites 5-hydroxyindoleacetic acid (5-HIAA) were measured by high performance liquid chromatography equipped with electrochemical detector (Acclaim, USA) and 25 cm $\times$ 0.5 cm I.D column. The mobile phase consisted of 0.15 mol/L chloroacetic acid-sodium hydroxide buffer solution (pH 4.0) and included 9 mmol/L sodium camphor sulfonate, 0.1 mmol/L EDTA, and 15% (v/v) methanol. The flow rate was maintained at 0.7 ml/min and potential was +700 mV. The detection sensitivity was 10 nA. The data were quantified using the area under the peaks and external standards. The concentrations of DA and its metabolites were expressed as μ g/g total protein.

Immunohistochemistry for tyrosine hydroxylase

Mice were deeply anesthetized with intraperitoneal injection of 10% chloral hydrate. Transcardial perfusion was performed with 200 ml normal saline followed by 400 ml 4% paraformaldehyde in 0.1 mmol/L PBS. The perfused brains were postfixed in 4% paraformaldehyde containing 20% sucrose for 2–4 hours, then placed into 30% sucrose phosphate buffer for 24 hours. The brain was then sectioned serially in coronal plane. Sections were made at 30 μ m thickness and stained with immunohistochemistry according to the instruction of ABC kit for tyrosine hydroxylase (Santa Cruz Biotechnology, USA).

Oxidative stress assays

Mice were decapitated, the brains were rapidly removed

and dissected on an ice-cold glass plate, and bilateral substantia nigra immediately were isolated from the cerebrum and weighed precisely. Samples were palced into 1.5 ml centrifuge tube, and superaudible cell disintegrator was applied to prepare 10% tissue homogenate, which was centrifugated at 100 000 $\times g$ for 60 minutes at 4°C. The supernatant corresponding to the cytosolic fraction was collected and kept at -20°C until analysis. Using detection kit (Nanjing Jiancheng Bioengineering Institute, China), the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX), and the content of malondialdehyde (MDA) were detected. The SOD activity was determined by hydroxylamine assay-developed from xanthine oxidase assay. The assay of GSH-PX activity and content of MDA were determined by colorimetry. Above assays were performed strictly according to the detection kit specification. Results were present as content or units of activity of per milligram brain tissue (wet weight).

Reverse transcription-PCR for DAT mRNA

According to mouse cDNA sequence, we used software Oligo 5.0 (Premier, Canada) to design the primers of DAT, which were as follows: upstream primer: 5'-ATGCTGCTCACTCTGGGTATC-3', downstream primer: 5'-TAGTG-TGGGGGTCTGAAGGTC-3', amplified fragment length was 404 bp. Beta-actin was used as internal standard. The primer sequences for beta-actin were as follows: upstream primer: 5'-CCTCTATGCCAACACAGTGC-3', downstream primer: 5'-GTACTCCTGCTTGCTGATCC-3', amplified fragment was 211 bp. Mice were decapitated after intraperitoneally injected with 10% chloral hydrate. Whole brains were removed rapidly from the skull. Then substantia nigra were disassociated in precooling 0.1 mol/L PBS. Total RNA was extracted with chloroform/isoamyl alcohol and precipitated with 70% ethanol. The resulting RNA was quantified spectrophotometrically, and then 0.5–1.0 g/L RNA was used to synthesize the cDNA. Reverse transcription (RT)-PCR reaction was done according to BcaBEST RNA PCR Kit (Takara, Japan) manual. Twenty-eight PCR cycles yielded a DAT fragment (404 bp) using an automated thermocycler (PTC100, MJ Research, Hercules, USA) with cycles of 94°C for 30 seconds, 61°C for 45 seconds, and 72°C for 1.5 minutes. Beta-actin (211 bp) was amplified from the same cDNA in a separate reaction and the same PCR cycle settings. In order to rule out the contamination of genomic DNA in total RNA, total RNA was PCR-amplified without reverse transcription directly. Experiment condition is identical to that of DAT. Then, 10 μ l PCR products from each group were electrophoresed on a 1.5% agarose gel and visualized with 1% ethidium bromide staining with a ultraviolet (UV) source. Semiquantitative analysis of sample mRNA Image Analyzer (Image Master VDS Pharmacia Biotech, Germany) was applied to scan the electrophoresis band and determine optic density of each band. Relative amount of sample mRNA were quantitated according to the ratio of optical density of sample band to that of corresponding beta-actin mRNA band.

Western blotting for DAT

Mice were decapitated, the substantia nigra were rapidly removed and dissected on an ice-cold glass plate. The samples were sonicated in 400 μ l of ice-cold lysis buffer (50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) and 0.02% sodium azide). Protein concentrations of the lysate were determined using the Bradford reagent (Bio-Rad, Hercules, USA). Samples (20 μ g protein) were loaded onto a 12% SDS-polyacrylamide electrophoresis gel for Western blotting analysis. After separation, the proteins were blotted on a nitrocellulose membrane and then shaken for 1 hour at room temperature in Tris-buffered saline (TBS) containing 0.1% Tween-20, 5% skim milk, and 0.2% bovine serum albumin (BSA). After shaking, the membranes were incubated for 1 hour at room temperature with primary antibodies (mouse monoclonal anti-DAT; Biotechnologies Santa Cruz; 1:1000) in TBS containing 0.1% Tween-20. The primary antibodies were detected by using a horseradish peroxidase (HRP)-coupled secondary antibody (anti-mouse; 1:1000), and the bound secondary antibodies were visualized using enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK). The band intensity of the detected protein was measured by densitometry. Actin was analyzed as a loading control.

Statistical analysis

Statistical analysis data were presented as mean $\pm$ standard deviation (SD). Student's *t*-test was used for the analysis comparing two different groups. Differences between the groups were compared by one-way analysis of variance (ANOVA). Statistical software SPSS 12.0 was applied. Statistical significance was accepted if $P < 0.05$.

RESULTS

Behavior changes of each group

There was no dystrophy in mice fed with equal volume saline. Mice treated with PQ (10 mg/kg) for four months showed more apparent behavior disorder, conspicuous trembling all over body at rest. In this study, ambulatory activity was expressed by horizontal activity. Changes in ambulatory activity after final treatment are shown in Figure 1. Total ambulatory activity was reduced by PQ and MPTP. Mice treated with PQ for four months showed hypoactive behavior compared with saline control group. There were no significant differences between PQ treated group and MPTP treated group.

PQ induced a decrease in striatal DA and its metabolites

Changes in levels of DA and its metabolites measured are shown in Table 1. The chronic systemic administration of PQ resulted in significant decreases of DA and its metabolites contents in the striatum. PQ at 10 mg/kg taken orally for four months significantly decreased DA content by 52%. Significant declines in DOPAC and HVA were also observed, while 5-HT and its metabolites showed no significant changes.

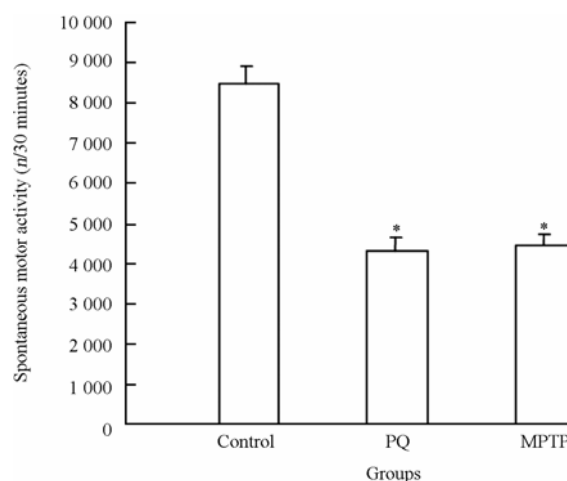


Figure 1. Locomotor activity during a 30-minute observation period was measured after PQ taken orally for four months. Horizontal activity was reduced by PQ. The differences observed were significant in both PQ and MPTP groups compared with the control group. * $P < 0.01$ vs control group.

Table 1. Changes of DA, 5-HT and metabolites in striatum of C57BL/6 mice (μ g/g wet tissue)

Groups	DA	DOPAC	HVA	5-HT	HIAA
Control	12.3 $\pm$ 1.07	1.30 $\pm$ 0.12	1.10 $\pm$ 0.12	0.51 $\pm$ 0.08	0.48 $\pm$ 0.06
MPTP treated	6.32 $\pm$ 0.78*	0.72 $\pm$ 0.10*	0.65 $\pm$ 0.09*	0.47 $\pm$ 0.09	0.45 $\pm$ 0.07
PQ treated	5.97 $\pm$ 0.85*	0.80 $\pm$ 0.11*	0.59 $\pm$ 0.10*	0.48 $\pm$ 0.08	0.46 $\pm$ 0.09

Compared with control group, * $P < 0.01$.

Numbers of TH-positive neurons were reduced following PQ treatment

In the saline group, there were many immunoreactive tyrosine hydroxylase (TH)-positive neurons in substantia nigra. TH-positive neuron cell counts in the substantia nigra were reduced after treatment with PQ compared with the control group. A similar result was observed in MPTP group (Figure 2). Quantitation of the immunoreactivity was achieved by using Quantimet 570 image processing and analysis system (Germany). Integral optical densities were 5439.1 $\pm$ 453.2, 2788.0 $\pm$ 367.5, and 2903.2 $\pm$ 337.1 in control group, PQ treated group, and MPTP treated group respectively, which were reduced by 48.7% and 46.6% in PQ treated group and MPTP treated group respectively, compared with control group ($P < 0.01$).

Oxidative stress assays

The activity of SOD and GSH-PX in substantia nigra were decreased markedly compared with that of control group ($P < 0.01$). The content of MDA of PQ and MPTP group were increased by 38% and 42% respectively ($P < 0.01$; Table 2).

Table 2. Changes of SOD, GSH-PX and MDA in substantia nigra of mice ($n=6$)

Groups	SOD (U/mg)	GSH-PX (U/mg)	MDA (nmol/mg)
Control	1.57 $\pm$ 0.132	1.63 $\pm$ 0.132	90.62 $\pm$ 13.61
MPTP treated	1.04 $\pm$ 0.116*	1.15 $\pm$ 0.109*	175.30 $\pm$ 19.27*
PQ treated	0.961 $\pm$ 0.113*	1.07 $\pm$ 0.117*	163.12 $\pm$ 15.33*

Compared with control group, * $P < 0.05$.

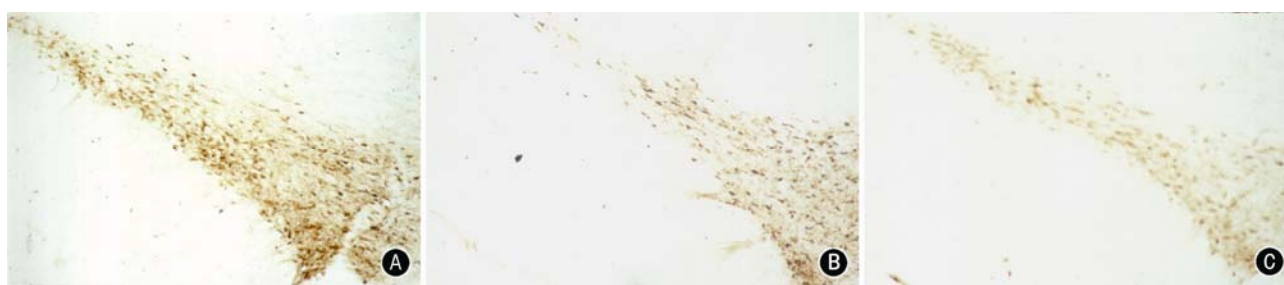


Figure 2. Immunohistochemical staining of TH in substantia nigra of mice (Original magnification $\times 40$). **A:** Control group. **B:** MPTP treated group. **C:** PQ treated group.

Detection of DAT mRNA expression

Agarose gel electropherogram of DAT mRNA and beta-actin mRNA (Figure 3)

After scanning and statistically analyzing, it was found that the mRNA expression of DAT was decreased from 0.87 ± 0.04 in control group to 0.48 ± 0.03 ($P < 0.01$) in PQ treated group and 0.45 ± 0.04 ($P < 0.01$) in MPTP treated group respectively. Above results indicated that the mRNA expression of DAT was decreased in substantia nigra when mice were given paraquat orally for four months ($P < 0.01$).

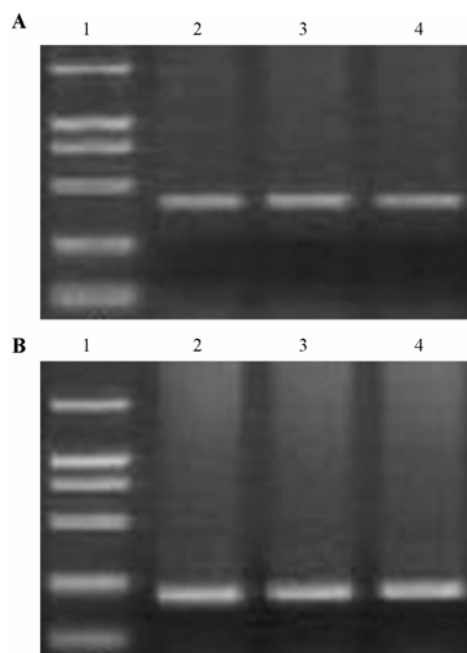


Figure 3. mRNA expression of DAT in substantia nigra of mice after PQ taken orally for four months. **A1** and **B1:** Markers (from above to below are 2000, 1000, 750, 500, 250, 100 respectively). **A2:** Control group. **A3:** PQ treated group. **A4:** MPTP treated group. **B2–B4:** mRNA expression of beta-actin.

Results of Western blotting of DAT and actin protein (Figure 4)

The quantitative analysis using actin as an internal reference showed a significant decrease of DAT protein levels, from 0.64 ± 0.04 in control group to 0.31 ± 0.03 and 0.35 ± 0.02 in PQ treated group and MPTP treated group, respectively (both $P < 0.01$). Above results indicated that the protein levels of DAT were significantly decreased in

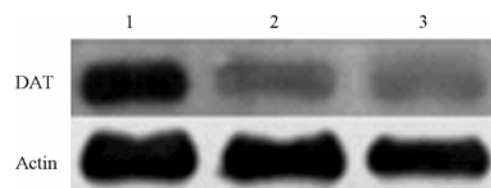


Figure 4. DAT protein level in substantia nigra of mice after PQ taken orally for four months. 1: Control group. 2: PQ treated group. 3: MPTP treated group.

substantia nigra when mice were given paraquat orally for four months ($P < 0.01$) compared with control group.

DISCUSSION

In recent years, there are a few investigation on PQ neurotoxicity which has suggested that this herbicide might be an environmental factor contributing to PD.³⁻⁵ Li et al⁸ and kang et al¹⁰ observed that injection of PQ into substantia nigra can change dopamine levels and behavior and produce neuronal loss. Our study showed that chronic taken orally with PQ may elicit a distinct decrease in the level of dopamine and activity of spontaneous motor in mice. Chronic PQ administration could induce mice to appear a series of the behaviors that were similar to those of PD.

TH is the the enzyme catalysing the rate-limiting step in dopamine synthesis. The expression of TH directly determine the DA level in brain.¹⁸ Some reports found that protein and mRNA of TH were reduced parallelly in substantia nigra of midbrain, which reflex the damage of dopaminergic neuron.^{19,20} We observed that TH positive neuron number and content of TH were reduced markedly after taken orally with PQ for four months.

Our studies showed that chronic exposure to herbicide PQ could induce neurochemical and behavioral changes in the adult mouse. It is of particular interest that the changes are similar to those observed after MPTP exposure. Along with these behavioral aberrations, both PQ and MPTP reduced the striatal content of DA and its metabolites without affecting 5-HT in the adult mice. Above results showed that PQ could result in degenerative effects on the nigrostriatal dopaminergic system in C57BL/6 mice, and thus provide a useful

environmental toxin-induced model of neurotoxicity with behavioral, pathological, and neurochemical features remarkably similar to those of PD.

Present study showed that the activity of SOD and GSH-PX substantia nigra were reduced compared with control group after taken orally with PQ for four months. Under normal physiological conditions, free radicals produced by metabolism can be inactivated by free radicals scavenging system. The antioxidant enzymes include catalase, glutathione peroxidase (GPX), and SOD. SOD catalyzes the dismutation of superoxide ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2), which protect against oxidative injury.^{21,22} GPX located in mitochondrion, convert H_2O_2 to H_2O , which prevent iron and H_2O_2 from producing more venenous $OH^{\cdot}$. Thus it showed the role of protecting cellular structures and telotism.²³ Present study showed that the activity of SOD and GSH-PX substantia nigra were reduced, which showed that antioxidative ability of dopaminergic neuron were impaired markedly. MDA is the end products of lipid peroxidation. MDA level can indirectly reflex the metabolic condition of oxygen radicals, thus it can serve as indices of oxidative damage.²⁴ Our study showed that the content of MDA in substantia nigra was increased compared with control group, which suggested that it occurred a severe oxidative stress state in substantia nigra. The decrease of SOD activity suggested the weakness of antioxidant system, while increase of MDA indirectly reflected the increase of oxygen radicals in dopaminergic neuron of substantia nigra. An increased oxidative stress leads to overconsumption of SOD and GPX. Thus antioxidative ability of dopaminergic neuron was reduced obviously, which induce consequent cell death of nigrostriata dopaminergic neurons.

Studies showed that DAT mRNA was solely expressed in dopaminergic neurons, so many investigators suggested that it should be regarded as the marker of dopaminergic neuron loss, which could reflex functional status of dopaminergic neurons.^{15,25,26} We observed that the mRNA and protein expressions of DAT in substantia nigra were decreased compared with control group when mice were taken paraquat orally for four months. These results suggested that DAT could be involved in the pathogenic mechanism of Parkinson's disease induced by PQ. On the other hand, these results also suggested that dopaminergic neuron of substantia nigra were impaired by PQ oral administration.

The significant reduction of striatal dopamine and its metabolites in PQ-treated mice suggested that PQ might preferentially target dopaminergic neurons rather than other neurons in the same region. The fact that PQ treatment was associated with a reduction in the TH-positive neurons and the expression of DAT provides evidence that PQ could produce preferential dopaminergic neurodegeneration in mice. Such toxicity on the dopaminergic nigrostriatal system seems

reasonable since the substantia nigra is rich in dopamine, which can undergo both enzymatic and non-enzymatic oxidation to produce free radicals. PQ-induced oxidative stress and consequent cell death of dopaminergic neurons can be responsible for the onset of the Parkinsonian syndrome.^{27,28} PQ can generate oxygen free radicals by redox cycling, and these radicals exert cytotoxic effects by disrupting mitochondrial complex I activity.^{29,30}

In conclusion, our data provide evidence that long-term repeated oral administration of PQ can selectively impair the nigrostriatal dopaminergic system of mice. These behavioral, morphological and biochemical findings suggest the possibility that PQ might play an important role in the pathogenesis of PD.

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