Appendix 1

Animal care and surgical procedures

Thirty female Wistar rats (230–280 g) were obtained from a breeding colony and housed under standard conditions for one week prior to the start of the study.

The rats were randomly divided into five groups (n=6): model, model + L-Dopa, model + APO, vehicle (DMSO), and control groups (Consort table). The control group received no intervention. After being anesthetized with an intraperitoneal injection of ketamine/xylazine, the rats in the first four groups were placed on a cotton bed secured to a stereotaxic frame followed by rotenone injection in the first three groups and DMSO injection in vehicle into the right ventral tegmental area (VTA) and SNc (1).

PD induction in animals

To establish the PD model, 0.5 µl of rotenone solution (12 mg/ml in DMSO, Abcam, UK/US) was infused into the right ventral tegmental area (VTA) (coordinates: AP: -5.0 mm; ML: 0.8 mm; DV: 8.1 mm) and the right SNc (coordinates: AP: -5.0 mm; ML: 1.8 mm; DV: 8.3 mm) at a flow rate of 0.2 µl/min. After the injection, the needle was kept in place for an additional five minutes to ensure complete diffusion of the solution (1). For the vehicle group, 0.5 µl of DMSO was infused into the right VTA and SNc using the same procedure. Post-operatively, the rats were provided with ibuprofen and penicillin in their drinking water for 24 hours to minimize discomfort and prevent infection.

APO-Induced Rotation Behavior Test

To confirm the induction of the Parkinson's model, the APO-induced rotation test was performed by administering a subcutaneous injection of 1.5 mg/kg of APO on days 2, 7, 14, 21, and 28 post-rotenone injection. The test was monitored starting 30 seconds after the APO injection and continued for up to 2 hours on the specified days. Dopamine receptor stimulation by APO leads to increased activity in the denervated striatum on the injected side compared to the non-injected side, resulting in rotational behavior ipsilateral to the lesion (2).

Bar Test

The rat's forelimb was placed on a horizontal cylindrical wooden bar (25 cm in width, 45 cm in height, and 1 cm in diameter), and the rat was suspended on the bar. The duration for which the animals held both forelimbs on the bar was recorded for each rat. The test was repeated after six minutes, and the average hanging times were calculated for final analysis (3).

Forepaw stride length during walking

A washable fiberglass apparatus was used for this test, consisting of a tunnel (5.5 cm in width × 100 cm in length × 12 cm in height) connected to a dark box (10 cm in width × 20 cm in length × 18 cm in height). A vertical sliding door was placed at the junction between the tunnel and the box, which was closed immediately after the rats entered the box, preventing them from returning to the tunnel. Additionally, a horizontal sliding door was located at the top of the box to facilitate the removal of the rats. The forepaws of each rat were painted with red ink, and they were placed at the entrance of the tunnel, allowing them to walk straight along the paper-covered tunnel floor. The footprints left on the paper were analyzed to determine stride length and width for each rat.

Morpurgo test

The Morpurgo test is a three-step evaluation method commonly used to assess muscle stiffness. In the first step, the animals were placed on a flat table and were given a score of 0.5 if they moved in response to a mild touch. In the second step, the rat's right forelimb was placed on a 3 cm-high wooden platform. A score of 0.5 was assigned if the rat did not correct its position within 10 seconds. The same procedure was repeated for the left forelimb. In the final step, the rat's right forelimb was placed on a 9 cm-high wooden platform, leaving the rest of its body suspended. A score of 1 was recorded if the animal remained immobile for 10 seconds. The same experiment was conducted for the left forelimb (2, 3).

Histochemical analysis of the brain tissue

For histological analysis, at week 8 post-rotenone injection, the rats were sacrificed, and their brains were removed, fixed in 10% formalin, and embedded in paraffin. The paraffin-embedded tissues were then sectioned for IHC analysis of alph-synuclein and TH. Amyloid fibril deposition was studied through Congo red (CR) staining.

Immunohistochemistry assay

The sectioned tissues were deparaffinized at 55 ˚C for 1 hour and rehydrated through a graded ethanol series (100%, 95%, 80%, and 70%). After washing with PBS for 5 minutes, the sections were treated with 0.3% hydrogen peroxide (Sigma-Aldrich, USA) for 30 minutes to block endogenous peroxidases. Antigen retrieval was performed by boiling the sections in 0.01 M citrate buffer (pH 6.0) for 3–5 minutes. The slides were then allowed to cool, washed with PBS for 5 minutes, and incubated in Tris-buffered saline (TBS) containing 0.05% Tween 20 for 5 minutes.

The sections were incubated overnight at 4 °C with primary antibodies: anti-tyrosine hydroxylase (TH) (1:500, ab112, Abcam, USA) and anti-alpha-synuclein (1:100, ab6162, Abcam, USA).

Negative controls were prepared by omitting the primary antibodies. After washing with PBS for 5 minutes, the sections were incubated with secondary antibodies: anti-sheep IgG HRP-conjugated (1:500, Zist Tolid Razi Co) and anti-rabbit IgG HRP-conjugated (1:100, Santacruz) for the detection of alpha-synuclein and TH, respectively, for 60 minutes. The sections were then washed three times with PBS, each for 5 minutes, followed by the addition of 4 mg 3,3′-diaminobenzidine (DAB) to enable color development. Finally, the sections were dehydrated using a graded ethanol series (70%, 80%, 95%, and 100%), and coverslipped with mounting medium. Observations were made using a light microscope (Olympus BX51, Japan) equipped with a Canon EOS digital camera. For each group, four images were taken from the left and right SNc areas of each rat at 40X magnification, and the mean percentage of immunoreactivity for alpha-synuclein was measured using ImageJ software. Additionally, one image was captured from the aforementioned areas at 10X magnification for all groups, and the total number of TH-positive cells was counted on each side of the SNc using ImageJ software.

Congo red staining

The deparaffinized sections were rehydrated using a graded ethanol series (100%, 95%, 80%, and 70%). Following rehydration, the sections were washed with distilled water and treated with 1% Congo red (CR) solution (C6277-25G; SIGMA; Germany) in 50% ethanol for 5 minutes. Subsequently, the sections were transferred to a 0.2% potassium hydroxide solution in 80% ethanol for 5 seconds. After rinsing with distilled water, the sections were stained with hematoxylin for 1 minute, dehydrated through a graded ethanol series, mounted on glass slides, and cover slipped.

The CR-stained sections were assessed blindly by an expert pathologist and scored as negative, mild, or severe beta-plated formation.

Table 1: The table presented all significant differences between the various groups at all time points about right stride length. The differences among the groups at other time points were not significant. *, **, and *** indicate $p<0.05$, $p<0.01$, and $p<0.001$, respectively.

Tukey's multiple comparisons test	Significancy	Adjusted P Value
Week 1		
Control vs. Model	***	0.0002
Control vs. Model + L-Dopa	**	0.0095
Control vs. Model + Apomorphine	**	0.0012
Vehicle vs. Model	***	0.0001
Vehicle vs. Model + L-Dopa	**	0.0084
Vehicle vs. Model + Apomorphine	**	0.0011
Week 3		
Control vs. Model + L-Dopa	*	0.0118
Control vs. Model + Apomorphine	*	0.0487
Vehicle vs. Model + L-Dopa	*	0.0195
Week 5		
Control vs. Model	*	0.0218
Week 7		
Control vs. Model	**	0.0027
Vehicle vs. Model	***	0.0009
Model vs. Model + L-Dopa	*	0.0403
Model vs. Model + Apomorphine	***	0.0008
Week 8		
Vehicle vs. Model	*	0.0115
Vehicle vs. Model + L-Dopa	*	0.0187
Model vs. Model + Apomorphine	*	0.0171
Model + L-Dopa vs. Model + Apomorphine	*	0.0227

Table 2: The table presented all significant differences between the various groups at all time points about left stride length. The differences among the groups at other time points were not significant. * and ** indicate $p<0.05$, and $p<0.01$ respectively.

Tukey's multiple comparisons test	Significancy	Adjusted P Value
Week 1		
Control vs. Model	**	0.0031
Control vs. Model + L-Dopa	*	0.0373
Control vs. Model + Apomorphine	*	0.0274
Vehicle vs. Model	**	0.0017
Vehicle vs. Model + L-Dopa	*	0.0326
Vehicle vs. Model + Apomorphine	*	0.0242
Week 7		
Vehicle vs. Model	*	0.0162
Week 8		
Vehicle vs. Model	*	0.0219

Table 3: The table presented all significant differences between the various groups at all time points about bar test. The differences among the groups at other time points were not significant. *,**, ***, and **** indicate $p<0.05$, $p<0.01$, $p<0.001$, and $p<0.0001$ respectively.

Tukey's multiple comparisons test	Significancy	Adjusted P Value
Week 2		
Control vs. Model	**	0.0011
Control vs. Model + Apomorphine	*	0.0353
Vehicle vs. Model	*	0.0429
Week 3		
Control vs. Model	***	0.0005
Control vs. Model + L-Dopa	**	0.0028
Control vs. Model + Apomorphine	*	0.0494
Vehicle vs. Model	**	0.006
Vehicle vs. Model + L-Dopa	*	0.0216
Week 4		
Control vs. Model	***	0.0001
Control vs. Model + L-Dopa	**	0.0022
Control vs. Model + Apomorphine	*	0.0143
Vehicle vs. Model	*	0.0273
Week 5		
Control vs. Model	**	0.0016
Week 6		
Control vs. Model	****	<0.0001
Control vs. Model + L-Dopa	**	0.0014
Vehicle vs. Model	***	0.0002
Vehicle vs. Model + L-Dopa	*	0.019
Model vs. Model + Apomorphine	**	0.0061
Week 7		
Control vs. Model	***	0.0001
Vehicle vs. Model	*	0.0402
Model vs. Model + Apomorphine	***	0.0006
Week 8		
Control vs. Model	***	0.0004
Vehicle vs. Model	*	0.0276
Model vs. Model + Apomorphine	**	0.0012

References

1. Xiong N, Huang J, Zhang Z, Zhang Z, Xiong J, Liu X, et al. Stereotaxical infusion of rotenone: a reliable rodent model for Parkinson's disease. PloS one. 2009;4(11):e7878.

2. Björklund A, Dunnett SB. The amphetamine induced rotation test: a re-assessment of its use as a tool to monitor motor impairment and functional recovery in rodent models of Parkinson's disease. Journal of Parkinson's Disease. 2019;9(1):17-29.

3. Haddadi R, Eyvari-Brooshghalan S, Nayebi AM, Sabahi M, Ahmadi SA. RETRACTED ARTICLE: Neuronal degeneration and oxidative stress in the SNc of 6-OHDA intoxicated rats; improving role of silymarin long-term treatment. Naunyn-Schmiedeberg's Archives of Pharmacology. 2020;393(12):2427-37.