Supplementary information

Preventive effects of nano-graphene oxide against Parkinson's disease via reactive oxygen species scavenging and anti-inflammation

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MATERIALS AND METHODS

6-OHDA lesoned rat model

General anesthesia was performed via an intraperitoneal injection of a mixture of 35 mg/kg zolazepam and tiletamine (Virbac S.A., Carros, France) plus 5 mg/kg xylazine (Bayer, Leverkusen, Germany) prior to any surgical procedures (1). Twenty three rats received a unilateral injection of 18 μg of 6-OHDA (Sigma) in 6 μL of 0.9% saline with 0.1% ascorbic acid into the medial forebrain bundle (MFB). The right MFB coordinates were AP −2.2 mm, L +1.5 mm relative to the bregma, and V −8.0 mm from the dura, with the tooth bar at +4.5 mm (1). The toxin was automatically delivered at a rate of 1 μL/min. When the injection was completed, the needle was kept in place for 5 min to prevent any back flow. The animals were then randomly separated into two groups: a PD control group (n = 10; no treatment) and daNGO group (n = 13) where each rat was subjected to a daily daNGO injection for 5 days (10 mg/ml, 30 mg/kg, i.p.).

Apomorphine-induced rotation test

Apomorphine-induced rotation tests were performed in the experimental rats at 7 days after the 6-OHDA injections. Briefly, soon after apomorphine (0.25 mg/kg; Sigma) in sterile water was injected subcutaneously, the rat was tethered to a rotometer system (Panlab, Barcelona, Spain) (1). Ipsilateral and contralateral rotations were automatically counted for 45 min. The data were expressed as the net (contralateral − ipsilateral turns) average rotations per min (RPM).

Stepping test

Stepping tests were performed as previously described (1). Briefly, while the rat's hindlimbs and one of the forelimbs was held by the experimenter, both the contralateral and ipsilateral forelimbs were alternatively tested. The tests were performed on a moving treadmill (Jeung Do Bio & Plant Co., Seoul, Korea) at a rate of 0.18 m/s for 10 s. All experiments were video-recorded to count the number of adjusting steps. The tests were repeated twice in each session and averaged across the two trials.

Tissue processing

To fix the tissues, the rats received transcardiac perfusion with 0.9% saline containing heparin (Hanlim Pharm, Seoul, Korea), followed by 4% paraformaldehyde solution. Brain tissues were rapidly extracted and preserved in

4% paraformaldehyde for 24 h, followed by dehydration in 30% sucrose until they sank. The SN sections (AP, −4.8 to −6.0 mm, 40 μm thick) were collected using a cryostat (Leica, Wetzlar, Germany) and preserved in 0.08% sodium azide (Sigma) in PBS at 4℃.

Immunohistochemistry

Immunohistochemistry was performed as follows. The SN sections were washed in washing buffer (0.5% bovine serum albumin in PBS) and then incubated in a blocking solution for 2 h. The sections were then washed three times and incubated with the primary antibodies indicated below overnight. After washing three times, the sections were incubated with Alexa Fluor antibodies (1:1,000; Invitrogen, Carlsbad, CA) for 2 h. The fluorescent-labeled tissues were then cover-slipped. the following primary antibodies were used: rabbit anti-TH antibody (1:1,000; Abcam, Cambridge, UK), mouse anti-TH antibody (1:2,000; Sigma), and mouse anti-Iba-1 antibody (1:300; Abcam).

Imaging and statistical analysis

Rat tissue sections were imaged using confocal microscopy (Carl Zeiss, Oberkochen, Germany) and ZEN microscope software (Carl Zeiss). All statistical analyses were conducted by Prism Software (GraphPad, La Jolla, CA). A Kruskal-Wallis test with a Dunn's post-hoc test for multiple comparisons was utilized to analyze cell viability and the DCF-DA fluorescence intensity. To analyze the differences in time-dependent patterns in the stepping tests and apomorphine-induced rotation tests, two-way repeated measures ANOVA with a Bonferroni post hoc test were conducted. The counts for the TH and Iba-1 positive cells were assessed with a Student's *t*-test. Cell intensities were quantified using the ImageJ program (NIH, Bethesda, MD). All data are presented as a mean ± standard error.

1. Yoon HH, Nam MH, Choi I, Min J and Jeon SR (2020) Optogenetic inactivation of the entopeduncular nucleus improves forelimb akinesia in a Parkinson's disease model. Behav Brain Res 386, 112551