Neuroprotective effects of Suhexiang Wan on the in vitro and in vivo models of Parkinson's disease

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Abstract

OBJECTIVE: To examine the role of KSOP1009 (a modified formulation of Suhexiang Wan essential oil) in an animal model of Parkinson's disease (PD) induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydro-3-pyridine (MPTP) injection.

METHODS: Cell toxicity, apoptosis, and reactive oxygen species (ROS) levels were analyzed in the human neuroblastoma cell line SH-SY5Y. After that, changes in animal behavior and tyrosine hydroxylase (TH) protein levels in the substantia nigra (SN) of MPTP-injected mice were examined. Three different doses of KSOP1009 (30, 100, and 300 mg/kg, n = 8 for each group) were administered daily for 7 d before MPTP injection and 14 d after MPTP injection, totaling 21 d.

RESULTS: MPP+, the active metabolite of MPTP, decreased the viability of SH-SY5Y cells, whereas KSOP1009 alleviated MPP+-induced cytotoxicity. KSOP1009 (10 and 50 mg/mL) reduced MPP+-induced ROS generation compared with the control group. Treatment with 1 mM MPP+ increased the percentage of depolarized/live cells, whereas KSOP1009 intake at a dose of 10 mg/mL decreased the percentage of these cells. The mean latency to fall in the rotarod test was reduced in mice treated with MPTP compared with the control group. However, mice receiving three different doses of KSOP1009 performed better than MPTP-treated animals. MPTP-treated mice were more hesitant and took longer to traverse the balance beam than the control animals. In contrast, KSOP1009-treated mice performed significantly better than MPTP-treated mice. Furthermore, the KSOP1009-treated groups had a significantly higher number of TH-positive neurons in the lesioned SN and significantly higher expression of TH in the striatum than the MPTP-treated group. MPTP treatment strongly induced Jun-N-terminal kinase (JNK) activation,
whereas KSOP1009 suppressed MPTP-induced JNK activation. In addition, KSOP1009 intake reversed the decrease in the phosphorylation levels of cAMP-response element-binding protein in the brain of MPTP-treated mice. KSOP1009 also restored the decrease in dopaminergic neurons and dopamine levels in the brain of MPTP-treated mice.

CONCLUSION: KSOP1009 protected mice against MPTP-induced toxicity by decreasing ROS formation and restoring mitochondrial function.

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Keywords: Parkinson disease; 1-methyl-4-phenyl-1, 2,3,6-tetrahydropyridine; Mitochondria; Reactive oxygen species; Suhexiang Wan

INTRODUCTION

Parkinson’s disease (PD) is one of the most disabling chronic neurodegenerative diseases, with a major impact on older adults’ lives and society as a whole. Despite advances in therapeutic interventions, patients with PD present typical motor symptoms, including tremor at rest, rigidity, akinesia, bradykinesia, hypokinesia, and non-motor symptoms, including neuropsychiatric symptoms, autonomic dysfunctions, impaired sense, sleep disorders, and fatigue. PD is characterized by the progressive loss of dopaminergic neurons and dopamine (DA) depletion in the substantia nigra (SN) and the formation of alpha-synuclein-containing Lewy bodies. Several molecular changes are claimed to be involved in dopaminergic neurodegeneration in PD, including oxidative stress and inflammation. The progression of PD is associated with an increase in dopamine metabolism in the SN and production of oxidizable species, including DA-derived reactive oxygen species (ROS) and pro-inflammatory cytokine secretions that engage astrocytes and endothelial cells. The engagement of these cells induces autocrine and paracrine responses that amplify inflammation, leading to neurodegeneration. Moreover, multiple genes were found to be associated with mitochondrial dysfunction and oxidative stress in PD.

Currently available pharmacologic treatments for PD include levodopa, DA agonists, and atypical neuroleptics. However, these medications relieve symptoms but do not prevent disease progression. Future research efforts should move from symptom-alleviating to disease-modifying therapies because of the side effects of the prolonged use of these drugs.

Suhexiang Wan (SHXW), a Chinese traditional medicinal prescription (Storax pill), consists of 15 crude herbs. This prescription has been used orally for treating seizures, infantile convulsion, depressed Qi (spirit or general vitality), sudden loss of consciousness, stroke, and other dysfunctions. Our previous studies examined the effect of a modified formulation (KSOP1009) of the SHXW essential oil in a transgenic mouse model of Alzheimer’s disease. The intake of KSOP1009 improved Ab-induced memory impairment by inhibiting tau protein hyperphosphorylation and further attenuating JNK and p38 phosphorylation in the brain.

In the present study, we examined the role of KSOP1009 in human cells and an animal model of PD induced by MPTP injection. Cell toxicity, apoptosis, and ROS levels were analyzed in human neuroblastoma cells. After that, changes in animal behavior and the protein levels of tyrosine hydroxylase (TH) in the SN of MPTP-injected mice were evaluated.

MATERIALS AND METHODS

Preparation of KSOP1009 and choice of standard molecular markers

A modified recipe of SHXW known as KSOP1009 was prepared as described previously. The KSOP1009 extract was prepared by using 13 kg of the following herbs: Liquidambaris Storax (Hamamelidaceae) (voucher number 2009.05, 411 g), Myristicae Semen (Myristicaceae) (2009.06, 1642 g), Ligustici Rhizoma (Umbeliferae) (2009.07, 2189 g), Santalii Alba Lignum (Santalaceae) (2009.08, 821 g), Pipers Longi Fructus (Piperaceae) (2009.09, 2737 g), Eugenieae Fructus (Myrtaceae) (2009.10, 821 g), Typhae Pollen (Typhaceae) (2009.11, 1095 g), and roots of Salvia miltiorrhiza Bunge (Lamiaceae) (2009.12, 3284 g). The mixture was pulverized and extracted twice with 10 volumes of 30% ethanol at 85-90 °C with a reflux condenser for 3 h, filtered with a 50-μm filter, and concentrated by vacuum evaporation at 60 °C. The final yield was approximately 2.4-3.6 kg of the dried mixture (mean yield rate of 23.08%). All medicinal herbs used were purchased from Dong Yang herb Pharm. Co. (Seoul, Korea). The quality of each of the plant specimens was identified and authenticated by Professor Byung-Soo Koo (Korean Medical Hospital, Dongguk University). The dried material was stored at −80 °C until use. For cell culture, each plant was extracted separately with 30% ethanol and concentrated by vacuum evaporation at 60 °C. Tan shinone IIA, eugenol, and cinnamic acid, obtained from the roots of Salvia miltiorrhiza Bunge, flower buds of Eugenia caryophyllata Merrill et Perry, and resin of Liquidamber orientalis Miller, respectively, were chosen as standard molecular markers. The amount of tanshinone IIA, eugenol, and cinnamic acid in 1 g of KSOP1009 extract was 0.0533 mg (0.00533%), 0.659 mg (0.0659%), and 0.752 mg (0.0752%), respectively.

Cell culture and determination of cell viability

Human neuroblastoma SH-SY5Y cells were obtained...
from the American Type Culture Collection (Rockville, MD, USA) and cultured in Dulbecco’s modified Eagle minimum essential medium (DMEM) containing 10% fetal bovine serum (FBS), 100 unit/mL penicillin and 100 mg/mL streptomycin (HyClone Laboratories Inc., Logan, UT, USA). The effect of KSOP1009 on cell viability was assessed by pre-treating the cells with the indicated concentrations of KSOP1009 for 1 h and stressing them with 1 mM of 1-methyl-4-phenylpyridinium (MPP +) (Sigma-Aldrich, St. Louis, MO, USA) for 24 h. The treated cells were incubated with 1 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA) at 37 °C in a CO2 incubator for 3 h. The MTT medium was carefully aspirated, and the formazan dye was eluted with dimethyl sulfoxide (DMSO). The plate was shaken, and its contents were measured using a spectrophotometer (Versamax microplate reader, Molecular Device, Sunnyvale, CA, USA) at a wavelength of 570 nm.

**Determination of intracellular ROS**

Intracellular ROS generation was detected using a fluorometer. The ROS-sensitive dye 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA) enters cells passively, is converted to dichlorofluorescein diacetate (DCFH), reacts with ROS, and ultimately forms the fluorescent product dichlorofluorescin (DCF). The treated cells were incubated with 20 mM H2DCFDA for 30 min. The fluorescence intensity was measured at 480 nm excitation and 530 nm emission using a fluorescence microplate reader (SpectraMax Gemini EM, Molecular Device, Sunnyvale, CA, USA).

**Determination of mitochondrial depolarization**

SH-SY5Y cells were seeded on 12-well plates at a density of 1 × 10⁴ cells/well for 24 h. MPP + (1 mM) was added to the cells in the presence or absence of KSOP1009 (10 mg/mL), and the cells were incubated for 1 h. The treated cells were harvested with trypsin, washed with PBS, and collected by centrifugation at 400 × g for 5 min. After centrifugation, the supernatant was removed, and the cell pellets were stained with the Muse MitoPotential Assay Kit (Merck Millipore, MA, USA) for 25 min at 37 °C. Cell viability was analyzed using the Muse Cell Analyzer (Merck Millipore, MA, USA).

**Murine PD model**

Ten-week-old male ICR mice with a mean weight of 29.5 g were housed in groups of four animals under standardized conditions [room temperature, (22 ± 1) °C; relative humidity, 55% ± 1%; light-dark cycle, 12 h]. The experimental procedures were performed in accordance with the animal care guidelines of the National Institute of Health (NIH) and carried out with prior approval of the Institutional Animal Research Ethics Committee (Protocol No. 2013-0992). The reagent 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, 30 mg/kg, i.p.; Sigma) was administered to mice from the negative control group (n = 8) once per day for 5 d [5]. Three different doses of KSOP1009 (30, 100, and 300 mg/kg, n = 8 for each group) and one dose of L-DOPA as the positive control (10 mg/kg, n = 8) were administered for 7 d before MPTP injection and 14 d after MPTP injection, totaling 21 d. Sterile saline (0.9% NaCl) was administrated daily (n = 8) and served as the negative control.

**Rotarod test**

Motor coordination and balance were evaluated using a rotarod apparatus (MED Associates Inc., St. Albans, VT, USA), which consisted of a motor-driven rotating rod operating at different speeds. Animal behavior was evaluated three times a day for 3 consecutive days with the rotation set to 4 to 35 rpm. Therefore, the presented data were the means from nine trials for each animal. The permanence time was recorded as the duration for which each animal stayed on the rod. The time of permanence of each mouse on the rod was correlated with motor coordination and balance. There were no significant differences in the daily performance of each animal in this test.

**Challenging beam traversal test**

The effect of MPTP was evaluated by monitoring the motor coordination and balance of the animals on a challenging team apparatus. The challenging beam consisted of a one-meter long wooden beam covered with surgical tape and suspended 23 cm above a bench top. The beam was divided into four gradually narrowing sections leading to the mouse’s home cage. The widths of the four beam sections distanced 25 cm apart were 3.5, 2.5, 1.5, and 0.5 cm in decreasing order. All mice were pre-trained for two consecutive days to traverse the beam starting at the widest section and ending at the narrowest and most difficult section. On the third day, each animal made five attempts, and data on the number of slips, number of steps, and traverse time were individually collected.

**Pole test**

The pole test was used to assess movement disorders in PD mice. The animals were placed head-up on top of a vertical wooden pole (8 mm wide and 55 cm high) with a rough surface. The base of the pole was placed in the home cage. The animals placed on top of the pole turned downward and descended the pole back into their home cage. Each mouse was trained and accustomed to the test one day before the test day. The time for turning downwards (T-turn) and the total time for descending the pole (T-total) until each animal reached the cage floor with four paws were measured in a total of three attempts. A default value of 60 s was used for the mice unable to complete the test successfully.

**Immunohistochemical staining**

Animals were anesthetized intraperitoneally with chlo-
ral hydrate (300 mg/kg) on the last day of KSOP 1009 administration and were perfused transcardially using phosphate buffered saline (PBS: 0.05 M, 0.9% NaCl, pH 7.4) and 4% paraformaldehyde (pH 7.4) for brain isolation. The isolated brains were fixed in 4% paraformaldehyde overnight at 4 °C and kept in a 30% sucrose solution. The right hemisphere containing the entire SN was coronally cut into 40-mm thick sections with a cryomicrotome (Leica VT 1000S; Leica Instruments, Nussloch, Germany). Immunohistochemical staining was performed for free-floating sections using mouse monoclonal anti-TH (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary antibody for DA neurons. Vectastain ABC reagent (Vector Laboratories, Burlingame, CA, USA) was used as the secondary antibody. The 3,3′-diaminobenzidine staining method was used for visualizing TH proteins.

Western blot analysis

The striatal tissue was dissected and homogenized in lysis buffer containing 50 mM Tris-base (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% glycerol, 10 mM NaF; 10 mM Na-pyrophosphate, 1% NP-40, and protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 5 μg/mL aprotinin, and 5 μg/mL leupeptin). Tissue homogenates were centrifuged at 800 g for 5 min at 4 °C. The supernatants were centrifuged at 3000 × g and used for Western blot analysis. Cell lysates were separated by 15% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked with 5% skim milk in TBS-T for 1 h and probed with rabbit anti-phospho-CAMP response element-binding protein (p-CREB), anti-CREB, anti-phospho-JNK, anti-JNK (1:1000; Cell Signaling Technology, Beverly, MA, USA) and mouse anti-TH (1:1000; Chemicon; Tenecula, CA, USA) overnight at 4 °C, and then incubated with HRP-conjugated secondary antibodies for 1 h. Immunoreactive bands were detected using the ECL system (Thermo Fisher Scientific, USA). Band images were visualized using Molecular Imager ChemiDoc XRS’ (Bio-Rad, Hercules, CA, USA), and band intensity was measured using Image Lab™ software version 2.0.1 (Bio-Rad). For internal standards, membranes were stripped and reprobed with mouse anti-β-actin. The relative density of each band was normalized against that of β-actin.

Ultra-high performance liquid chromatography (UHPLC) analysis of DA levels in the brain striatum

The collected striatal samples were homogenized in ice-cold water at a concentration of 20 mL/g using a homogenizer. One-hundred microliters of ice-cold 250 mM formic acid solution was added to the brain homogenates (left striatum), and the samples were mixed by vortexing for 1 min. Subsequently, the homogenates were centrifuged at 13 200 rpm for 10 min at 4 °C, and 10 μL of the supernatant was injected into a UHPLC-MS/MS system.

UHPLC-MS/MS-MRM

Chromatographic separation was achieved using a Waters ACQUITY UPLC BEH C18 column (100 ’ 2.1 mm, 1.7 μm) combined with a Waters UPLC H-Class system (Waters, Milford, MA, USA). Gradient elution was performed with two solutions [0.1% HFBA in water (mobile phase A) and methanol (mobile phase B)] as follows: equilibration with 0% of B for 0.0–1.5 min, 0%-50% of B for 1.5-7.0 min, 50%-95% of B for 7.0-7.1 min, 95% of B for 7.1-8.0 min, 95%-0% of B for 8.0-8.1 min, and re-equilibration with 0% of B for 8.1-10.0 min. The flow rate and injection volume were set at 200 μL/min and 10 μL, respectively. All analytes were detected using an API 3200 triple quadrupole mass spectrometer system (Applied Biosystems Inc., Foster City, CA, USA). Samples were analyzed by Turbo V electro spray ionization in the positive ion multiple reaction monitoring (MRM) mode. The mass spectrometric conditions were set as follows: ion spray voltage at 5200 V, curtain gas at 20 psi, nebulizer gas at 50 psi, auxiliary gas at 50 psi, temperature at 500 °C, and collision-activation dissociation gas at 5. Quantifier and qualifier MRM transitions were selected as the most abundant product ion and second most intense product ion, respectively. Data acquisition and analysis of all MRM chromatograms were performed using Analyst software version 1.5.2 (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

All statistical analyses were conducted with SPSS (ver. 19, Somers, NY, USA). Values are expressed as mean ± standard error of mean (SEM). Data were analyzed by one-way analysis of variance, and differences between means were analyzed using Dunnett’s test or Tukey-Kramer’s multiple comparison test. Differences were considered significant at P < 0.05.

RESULTS

Effects of KSOP1009 on cell viability and ROS production of MPP+-treated cells

The cytotoxic effect of different concentrations of KSOP1009 on SH-SY5Y cells was determined using the MTT assay. KSOP1009 was not cytotoxic at any of the tested concentrations (Figure 1A). On the contrary, MPP + was cytotoxic at 1 mM at a cell viability of 60% compared with the control (Figure 1B). However, the viability of cells treated with 10, 50, and 100 mg/mL KSOP1009 was significantly higher than that of MPP+-treated cells, indicating that MPP + decreased the viability of human neuroblastoma SH-SY5Y cells, whereas KSOP1009 alleviated MPP+-induced cytotoxicity. DCFDA was used to investigate the effect of KSOP1009 on MPP+-induced ROS production in SH-SY5Y cells. The ROS levels were increased
two-fold \((P < 0.05)\) in the MPP\(^{+}\)-treated group compared with the control group, whereas treatment with 10 and 50 mg/mL of KSOP1009 reduced the percentage of ROS\(^{+}\) cells to 10\% (baseline) and 15\%, respectively (Figure 1C).

**Protective effect of KSOP1009 on the MPP\(^{+}\)-induced mitochondrial dysfunction of SH-SYSY cells**

To determine whether MPP\(^{+}\) is associated with the mitochondria-mediated apoptotic pathway, MPP\(^{+}\)-treated SH-SYSY cells in the presence or absence of KSOP1009 were stained with the Muse MitoPotential Assay Kit, and cell viability was analyzed using the Muse Cell Analyzer. Treatment with 1 mM MPP\(^{+}\) increased the percentage of depolarized/live cells, indicating loss of mitochondrial membrane potential (Figure 2). However, KSOP1009 at a dose of 10 mg/mL decreased the percentage of depolarized/live cells.

**Restoration of behavioral deficits of MPTP-treated mice by KSOP1009**

The mean latency to fall in the rotarod test was reduced in MPTP-treated mice compared with control group treated with sterile saline (Figure 3A, \(P < 0.01\)). In contrast, mice treated with KSOP1009 (100 and 300 mg/kg) had a performance similar to that of the positive control group (MPTP+L-dopa) and performed better than the MPTP-treated group (Figure 3A).

21 d after KSOP 1009 treatment, the treated mice had a significantly higher number of slips on the challenging beam than those of the control group (Figure 3B). In addition, MPTP-treated mice were more hesitant and took longer to traverse the beam before returning to the home cage than the control group (Figure 3C). However, mice treated with three different doses of KSOP1009 performed significantly better than the MPTP-treated group, and the therapeutic effects in the former group were similar to those of the positive control group.

MPTP-treated mice were slower in both pole test parameters (time to turn and total time) compared with the control group (Figure 4). KSOP1009 reversed these deficits, and the performance of the KSOP1009-treated groups was similar to that of the positive control group (MPTP+L-dopa).

**Protective effects of KSOP1009 on the MPTP-induced degeneration of TH-positive cells**

The effects of KSOP1009 in MPTP-treated mice were assessed by the immunohistochemical analysis of TH-positive DA neurons in the SN. Photomicrographs of TH-positive neurons in the SN are shown in Figure 5. The MPTP-injected lesioned SN had significantly fewer TH-positive neurons compared with the intact SN in the control group \((P < 0.05)\). In contrast,
MPTP only. MPTP + L-dopa was used as a positive control. The dose of L-dopa used was (10 mg/kg, 21 d).

The data represent the mean ± standard error of mean. *P < 0.01 vs MPTP only, **P < 0.05 vs MPTP only, ***P < 0.001 vs MPTP only. MPTP + L-dopa was used as a positive control. The dose of L-dopa used was (10 mg/kg, 21 d).

the KSOP1009-treated groups had a significantly higher number of TH-positive neurons in the lesioned SN compared with the MPTP-treated group (P < 0.05).

TH protein expression in the striatum assessed by Western blotting was significantly decreased in MPTP-treated mice compared with the control group (Figure 6, P < 0.05). The administration of KSOP1009 (100 and 300 mg/kg) to MPTP-treated mice reversed the decline in the TH levels (Figure 6, P < 0.05).

Changes in the levels of signaling molecules after KSOP1009 administration in MPTP-treated animals

The levels of phosphorylated JNK (p-JNK) were significantly increased in the brain striatum of the MPTP-treated group compared with the control group (Figure 7A, P < 0.001). Of note, KSOP1009 treatment (30 and 100 mg/kg) significantly reversed the increase in the p-JNK levels induced by MPTP (Figure 7A, P < 0.01). In addition, MPTP reduced the levels

Figure 5 Effect of KSOP1009 on the survival of TH-positive cells in the substantia nigra of MPTP-treated mice

A-D: immunohistochemical staining of TH-positive neurons. A: normal group (treated only with physiological saline, 21 d); B: MPTP (30 mg/kg, 5 d)-treated group; C: MPTP (30 mg/kg, 5 d)-treated with KSOP1009 (100 mg/kg, 21 d) -treated group; D: MPTP (30 mg/kg, 5 d)-treated with KSOP1009 (300 mg/kg, 21 d) -treated group. E: Number of TH-positive neurons in the substantia nigra of mice. TH: tyrosine hydroxylase; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. The data represent the mean ± standard error of mean. *P < 0.05 vs control, **P < 0.05 vs MPTP only. Each bar corresponds to 100 μm.
of phosphorylated CREB compared with the untreated control group (Figure 7B, \( P < 0.05 \)). In contrast, KSOP1009 treatment (30, 100, and 300 mg/kg) significantly increased the levels of phosphorylated CREB in the brain of MPTP-treated mice (Figure 7B).

**DA levels in the striatum of MPTP-treated mice after KSOP1009 treatment**

The DA levels in the striatum of KSOP-treated mice were evaluated by UHPLC (Figure 8). The DA levels were significantly decreased in the striatum of the MPTP-treated group compared with the control group (\( P < 0.001 \)). In contrast, KSOP1009 treatment significantly increased the DA levels in the striatum of the MPTP-treated animals (\( P < 0.001 \)).

**DISCUSSION**

It is essential to create an animal model to study the pathogenesis of PD and develop new alternative therapies. Considering that developing an animal model that expresses the complete spectrum of PD is unfeasible, the MPTP-induced PD model used in the present study may help elucidate specific aspects of the disease because MPTP may lead to the rapid degeneration of dopaminergic neurons.\(^{12}\)

In the present study, KSOP1009 alleviated the MPP+–induced cytotoxicity of human neuroblastoma cells, decreased ROS generation, and reduced the percentage of depolarized cells. In addition, KSOP1009 improved motor performance and coordination and prevented the decrease in the TH protein levels by accelerating the recovery of TH-positive neurons in the lesioned SN.

The traditional prescription of SHXW was changed to KSOP1009 (with eight medicinal plants) to improve PD-like symptoms. In KSOP1009, the methanolic extract of Cnidium officinale has scavenging activity against free radicals and antioxidant capacity.\(^{15}\) Moreover, tanshinone IIA, one of the key components of Salvia miltiorrhiza Bunge, has neuroprotective properties.\(^{14,15}\) In our previous study, we reported that KSOP1009 presented a stronger neuroprotective effect than each herb in isolation.\(^{10,11}\)

In this study, the sub-acute toxicity of MPTP was eval-
Mitochondria are organelles that regulate apoptotic cell death when subjected to stress; therefore, the change in mitochondrial membrane potential is considered an early sign of apoptosis.\textsuperscript{21} Mitochondrial depolarization may be pivotal to apoptosis-related events, including the release of cytochrome c from mitochondria, activation of caspase-3, formation of apoptosome (constructed by apoptotic protease activating factor-1 and caspase-9 in the cytosol), and DNA fragmentation.\textsuperscript{22} Our results demonstrated that MPP\textsuperscript{+} induced mitochondrial damage by significantly depolarizing them. Although mitochondrial depolarization is critical, the subsequent increase in the levels of ROS generated by mitochondria plays an important role in the degeneration of dopaminergic neurons. It is known that the onset of mitochondrial damage due to the disruption of its structural integrity causes leakages in the electron transport chain and consequently increases ROS levels.\textsuperscript{23,24} This result confirms that intracellular ROS production occurs downstream of mitochondrial depolarization. The decrease in the mitochondrial membrane potential and ROS levels was abolished by KSOP1009. It is of note that KSOP1009 reversed the TH protein levels in the striatum to baseline levels, which corroborated the presence of TH-positive DA neurons in the SN. Moreover, KSOP1009 treatment reversed the decrease in DA levels in the brain of MPTP-treated mice. This result suggests that the function and number of dopaminergic neurons were restored by KSOP1009 treatment. In addition, the recovery of TH protein levels by KSOP1009 after 14 d of MPTP treatment indicates that complete neuronal cell death did not occur in the striatum.

Apart from the inflammatory reaction, most of the neurodegenerative process in PD is attributed to disturbances in the structure of the basal ganglia, which include the SN. The SN is composed of the pars reticulata and pars compacta, the latter being the main afferent compartment of dopaminergic innervations to the caudate-putamen of the dorsal striatum.\textsuperscript{25} Damage to the pars compacta or caudate-putamen causes noticeable deficits in motor function. The impaired motor performance and coordination of MPTP-treated mice in the rotarod test, challenging beam traversal test, and pole test mimicked the limited joint mobility and reduced motor performance in patients with PD. Moreover, the activity of signaling molecules such as JNK and CREB was affected by MPTP treatment, and KSOP1009 reversed the effect of MPTP, indicating that the impairment of neuronal cells is responsible for the locomotor dysfunction observed in MPTP-treated mice. Our findings demonstrated that the use of 30 mg/kg MPTP for 5 d induced the partial reproduction of PD phenotypes, including decreased motor coordination, mitochondrial dysfunction, increased ROS levels, and neurodegeneration of the TH-positive cells located in the striatum. In addition, severe neuropathological changes were demonstrated by histochemical
staining, which revealed the selective loss of TH-positive DA neurons in the SN. The selective vulnerability of the dopaminergic neurons of the SN to MPTP is not fully understood but is believed to be largely due to the increased production of ROS in DA neurons due to DA metabolism. We used three independent behavior tests to demonstrate that KSOP1009 could partially prevent MPTP-induced motor deficits. There was a significant reduction in the levels of oxidative stress and mitochondrial dysfunction after KSOP1009 intake, and these events underlie the neuropathological changes in PD. Furthermore, KSOP1009 treatment significantly attenuated the MPTP-induced decrease in the levels of TH proteins in the striatum.

In conclusion, our results demonstrated that KSOP1009 intake protected against MPTP toxicity in mice. The short-term treatment following the subacute phase of injury reversed the decrease in the TH protein levels and degeneration of DA neurons in the striatum. The mechanism of neuroprotection of KSOP1009 may be associated with a decrease in DA turnover, resulting in a reduction in ROS formation and restoration of mitochondrial function.

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