Beneficial effect of transient desflurane inhalation on relieving inflammation and reducing signaling induced by MPTP in mice

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Abstract

Objective: To determine if the beneficial effects of transient desflurane application mitigates inflammation and decrease associated signaling induced by 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) in mice.

Methods: Mice were induced to develop Parkinson’s disease (PD) by intraperitoneal injection with MPTP for 20 consecutive days, and validated mice were randomly allocated to four groups. Collected samples from euthanized mice were designated for the following analyses: 1) immunohistochemical staining for positive dopaminergic neurons in the substantia nigra and striatum, 2) immunofluorescence staining for ionized calcium binding adaptor molecule-1 (Iba1) and glial fibrillary acid protein (GFAP), and 3) western blotting for p38, p-p38, toll-like receptor 4, and tumor necrosis factor (TNF-α).

Results: The inhalation of desflurane for 1 hour ameliorated locomotory dysfunctions of PD mice by recovering the loss of Iba1- and GFAP-positive dopaminergic neurons, deactivating microglial cells and astrocytes, and decreasing the amounts of inflammatory cytokines (TNF-α).

Conclusions: These findings suggest that transient desflurane inhalation may provide some benefits for PD through ameliorating inflammation and enhancing locomotor activity.
Keywords
Desflurane, MPTP, neuroinflammation, Parkinson’s disease, toll-like receptor, signaling

Introduction
Parkinson’s disease (PD) is one of the most common neuro-degenerative disorders plaguing older people worldwide. The median age-standardized annual incidence rate of PD is 14 per 100,000 people in the total population in high-income countries and 160 per 100,000 in the elderly population aged 65 or older.1 PD is attributed to the severe loss of dopamine-positive neurons with the depletion of dopamine in the striatum, which extends to the substantia nigra pars compacta with nerve fibers that project to the striatum.2 The neurodegeneration in PD is a selective, aggressive, and irreversible process that leads to major motor disorders, and its clinical symptoms can be classified as akinesia/bradykinesia, rigidity, and resting tremors. Other disturbances include anxiety and depression, apathy, cognitive deficits, visual and olfactory defects, and insomnia.2–4 A plethora of leading causes have been proposed. The focus was initially placed on the dysfunction of mitochondria and then shifted to the loss of dopaminergic neurons. Glial cells, astrocytes, and inflammation mediated by toll-like receptors (TLRs) are also involved.5,6

Different biomarkers have been screened but remain to be validated. In addition, the 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP), a potent dopaminergic neurotoxin, induced mouse model remains a recommended animal PD model, and it is useful to evaluate potential candidates for treatment.4 MPTP, a toxic byproduct from the synthesis of the opioid drug desmethylprodine that targets dopaminergic neurons, produces irreversible PD-like symptoms in animals that mimic PD in humans. The MPTP injection-induced mouse model displays unique biological traits, including dopaminergic neuronal loss in the substantia nigra and dopamine depletion in the striatum. Moreover, it has several advantages for exploring molecular landscapes and provides a platform to discover drug candidates.7 Recently, volatile anesthetic agents, such as sevoflurane and desflurane (DES), have been extensively studied, providing encouraging and promising results. Because of their stable physical properties, rapid anesthesiologic induction, and minimal inhibition of circulation, these anesthetics have been demonstrated to provide great neuroprotection.8 In addition, DES is considerably safer than sevoflurane in promoting learning and memory because sevoflurane may open mitochondrial permeability transition pore, reducing mitochondrial membrane potential.8 Here, using a mouse PD model induced by MPTP, we aimed to investigate if DES inhalation alleviates dopaminergic neuronal damage by deactivating microglial cells and astrocytes, reducing associated inflammatory signaling, and recovering motor function.

Materials and methods

Animals
All C57BL/6 SPF mice were purchased from the Experiment Animal Center of
Jinling Hospital, Nanjing Medical University (Nanjing, China). All mice (male, aged between 20–24 weeks, weighing 26–30 g) were individually housed with animal enrichment at a constant room temperature (20–25°C) and moisture of 40% to 70% under a 12:12 hour light:dark cycle with ad libitum access to food and water. The animal protocols used in the study followed the Guide for the Care and Use of Laboratory Animals (IACUC No. YYZD 201400, 1 July 2019) by the National Institutes of Health and were approved by the Animal Care and Use Committee of Nanjing Medical University. We ensured good welfare and humane treatment for experimental animals, reduced the number of experimental animals used in experiments, and minimized their suffering. The reporting of this study conforms to ARRIVE 2.0 guidelines.9

**PD model development and grouping**

In total, 100 male mice were prepared for intraperitoneal injection (I.P.) with MPTP (4 mg/kg, Sigma, St. Louis, MO, USA) for 20 consecutive days, whereas mice in the control group were injected (I.P.) with saline (4 mL/kg).10,11 All mice that survived were screened by the pole test and validated for the development of the PD model. According to the DES (Baxter Healthcare Corporation, Deerfield, IL, USA) inhalation times of 0, 1, 2, and 4 hours, PD mice were randomly divided into four groups (MPTP + DES0, MPTP + DES1, MPTP + DES2, and MPTP + DES4). The concentration of DES administered was 7.5% (carrier gas: 100% oxygen, flow rate at 2 L/min), equivalent to a minimum alveolar concentration of 1.0 in mice.12 Another author recommended 7.5% DES and 100% oxygen for 2 hours.13 In contrast, mice in the saline group were treated with 100% oxygen for 4 hours. Mice were placed into a self-designed and transparent plastic box (18 cm × 18 cm × 20 cm) for anesthesia and concomitantly connected to an oxygen tank and DES tank with a Datex Ohmeda Capnomac Ultima Anesthesia Monitor (Datex-Ohmeda, Capnomac Ultima, Helsinki, Finland). The first injection of MPTP was defined as Day 1, and all behavioral studies were performed between 13:00 and 17:00 under dim lighting from Day 22 to Day 24. At the end of the study on Day 25, mice were euthanized for sample analysis.

**Experimental reagents**

The antibodies used in this study are provided in Table 1.

| Antibody | Company | Catalog number | Animal species | Dilution of primary antibody | Dilution of secondary antibody |
|----------|---------|----------------|----------------|-------------------------------|-------------------------------|
| p38      | Abcam   | ab170099       | Rabbit         | 1:1000                        | 1:3000                        |
| p-p38    | Abcam   | ab31828        | Rabbit         | 1:1000                        | 1:3000                        |
| TLR4     | Servicebio | GB13187       | Rabbit         | 1:1000                        | 1:3000                        |
| TNF-α    | Servicebio | GB13188-1     | Rabbit         | 1:1000                        | 1:3000                        |
| Iba1     | Abcam   | ab15690        | Rabbit         | 1:500                         | 1:200                         |
| GFAP     | Abcam   | ab7260         | Rabbit         | 1:500                         | 1:200                         |
| TH       | Cell Signaling Technology | 58844         | Rabbit         | 1:100                         | 1:600                         |

TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor-alpha; Iba1, ionized calcium binding adaptor molecule-1; GFAP, glial fibrillary acid protein; TH, tyrosine hydroxylase.

**Table 1. Antibodies.**
**Pole test**

The pole test is used to detect bradykinesia and motor coordination in PD mice.14,15 The mice were placed facing upwards at the top of a wooden, rough surface pole (55 cm long and 0.8 cm in diameter), and the time that the mouse spent traversing from the top of the pole and moving downward to the ground was recorded. The mice were trained in two sessions on consecutive days before MPTP or saline injections to turn downward and traverse along the pole until they landed on the ground. Because of dopaminergic neuronal destruction and dopamine depletion, the PD mice could not turn downward along the pole and fell down from the top of the pole. After 20 days of consecutive injection with MPTP, the mice were tested, and the total amount of time was recorded. Three trials were performed with each mouse, and the median was taken across trials. Times were limited to 120 s.15

**Hanging-wire test**

Neuromuscular strength was examined by the hanging-wire test,16 with modifications. Each mouse was hung on a wire (5 mm in diameter) connected to the soft board at the bottom. The distance between the top and the bottom was 40 cm. The latency from the beginning of the test until the mouse hung with at least two limbs on the wire was timed. The mouse was allowed three attempts to hang for a maximum of 180 s per trial, and the average was obtained. The longest latency was also recorded. The interval between two trials was set to 15 minutes.

**Open field test**

An open field test was performed in a chamber (40-cm length × 40-cm width × 40-cm height) using the XR-SuperMaze Animal Behavior Video Tracking and Analysis System (Shanghai Xinruan Information Technology Co. Ltd., Shanghai, China). The floor of the chamber was divided into four grids. During the test, the environment was kept quiet and dark to avoid interference or distraction. Each mouse was placed in the center of the experimental apparatus immediately prior to testing and allowed to explore for 5 minutes. The trajectory of each mouse, including the total distance traveled and the time spent in the center area, was recorded by the computer. Between each test, 75% alcohol was applied to clean and remove the odors of the previous mouse to avoid odor interference.

**Immunohistochemistry**

Mice were overdosed with sodium pentobarbital (60 mg/kg, I.P.) and decapitated. The brain was removed, immersed in the same 4% paraformaldehyde fixative for 72 hours, and cryopreserved in 30% sucrose in 0.1 M phosphate buffer saline (PBS) for 48 h at 4°C. Immunohistochemistry was performed as described previously on free-floating cryomicrotome-cut sections (10 µm) encompassing the entire brain.17 Sections were incubated with a polyclonal antibody against tyrosine hydroxylase (TH) (1:100 diluted in tris-buffered saline [TBS] with 0.15% Triton X-100 and 0.5% bovine serum albumin) for 1 hour at 37°C and overnight at 4°C. After three rinses in TBS, these were incubated for 2 hours at room temperature in goat anti-rabbit IgG serum diluted at 1:200 in TBS with 0.25% bovine serum albumin for removing signal noise background. They were then rinsed in TBS and incubated in rabbit peroxidase-anti-peroxidase complex diluted at 1:500 for 2 hours. After thorough rinsing, followed by treatment with 3, 3 diaminobenzidine tetrahydrochloride diluted in TBS and H2O2, slices were rinsed three times in TBS for 1 minute each, mounted on gelatin-coated slides, dried, dehydrated in graded concentrations of ethanol, cleared in xylene,
and coverslipped in Neoantelan (Polylabo, Strasbourg, France). Finally, the images were obtained using a NIKON microscope and processed with the NIKON NISELEMENTS Microscopic Image Processing System (Nikon Inc., Melville, NY, USA).

The number of TH-positive cells was counted in the substantia nigra, and the density of TH-positive fibers was calculated using a colorimetric method. Briefly, the content of TH-positive fibers in the striatum was evaluated by measuring the optical density of specific staining in five striatum sections from each mouse (four areas in each section) using ImageJ software (NIH, Bethesda, MD, USA). 

**Immunofluorescence staining**

For immunofluorescence staining, mice that completed different treatments were perfused with cold normal saline followed by 0.1 M PBS (pH 7.3) containing 4% paraformaldehyde under anesthesia. The brain was then removed, post-fixed in the same fixative for 4 hours, and cryoprotected for 24 hours at 4°C in 0.1 M PBS containing 30% sucrose. Serial coronal sections (30 μm) for each brain were obtained via a Leica cryostat (CM1800, Leica, Heidelberg, Germany) and collected serially into three dishes, each of which contained a complete serial section. Warmed sections were rinsed in 0.01 M PBS (pH 7.3) three times (10 minutes each) and blocked with 2% goat serum in 0.01 M PBS containing 0.3% Triton X-100 for 1 hour at room temperature. The sections were incubated overnight at 4°C with primary antibodies (ionized calcium binding adaptor molecule-1 [Iba1] and glial fibrillary acid protein [GFAP]) using the dilutions in Table 1. Microglial activation was determined as Iba1 positivity by immunofluorescence staining, whereas astrocyte activation was indicated by GFAP positivity. The sections were then washed three times in 0.01 M PBS (10 minutes each) and incubated with corresponding secondary antibodies for 2 hours at room temperature. Images were captured under a confocal microscope (LSM700, Zeiss, Jena, Germany), and fluorescence intensities were semi-quantitatively determined with Image-Pro Plus (Media Cybernetics, Rockville, MD, USA).

**Western blotting**

Mice were euthanized by cervical dislocation. The hippocampus was dissected and homogenized in sodium dodecyl sulfate sample buffer with a mixture of proteinase and a phosphatase inhibitor cocktail. The rest of the routine procedure was performed as described by previous reports. In the present study, several major signaling pathways were probed, including p38, p-p38, TLR4, and tumor necrosis factor (TNF)-α. β-actin was used as a control. The colorimetry of each band on the transferred membrane was quantitatively measured by Image J.

**Statistical methods**

All quantitative data were expressed as the mean ± standard error, examined by the F test, and showed a normal distribution. The statistical differences among groups were determined with a one-way analysis of variance followed by Bonferroni correction post-testing using IBM SPSS Statistics for Windows, Version 24.0 (IBM Corp., Armonk, NY, USA). The changes were considered statistically significant if the P-value was less than 0.05.

**Results**

**Development and validation of PD mouse model by intraperitoneal injections of MPTP for 20 consecutive days**

A chronic PD mouse model was generated by consecutive injections with MPTP (I.P.,...
4 mg/kg) for 20 days. One hundred male C57BL/6 mice were used for the development of the PD model, but four mice died, leaving 96 mice after model completion. As shown in Figure 1, the traversing times along the pole for mice in the MPTP groups were significantly longer by almost 3-fold than those for mice in the saline group ($P < 0.01$).

**Motor function phenotype of PD mice induced by MPTP intraperitoneal injection ameliorated by transient DES inhalation (1 hour)**

The hanging-wire test and open field test are frequently used to assess locomotor activity, neuromuscular strength, and coordination. Compared with mice in the saline group, MPTP-induced PD mice showed dramatically reduced times in the hanging-wire test ($P < 0.01$, Figure 2a). However, the hanging-on times of mice in the MPTP + DES$_1$ group were longer than those in the saline group ($P < 0.01$). The hanging-on times of mice from all DES inhalation treated groups were considerably lower than those of mice in the saline group ($P < 0.01$).

In terms of the open field test, the total exploring or tracking time was shown in Figure 2b, and the time spent in the central area was provided in Figure 2c. Compared with the total tracking distance of mice in the saline group, the total distances of mice in the MPTP + DES$_0$ group were significantly reduced ($P < 0.01$). With DES inhalation for 1 or 2 hours but not 4 hours, the total tracking distances and time spent in the central area for mice in the MPTP + DES$_1$ group and MPTP + DES$_2$ group were considerably longer than those for mice in the MPTP + DES$_0$ group ($P < 0.01$ and $P < 0.05$, respectively). However, the total tracking distances in both the MPTP + DES$_1$ group and MPTP + DES$_2$ group were shorter than those in the saline group (all $P < 0.01$).

**Reduced TH-positive cells in the substantial nigra from MPTP-induced PD mice were preserved by transient DES inhalation (1 hour)**

As shown in Figure 3a and b, the number of TH-positive neurons in the substantial nigra was lower in the MPTP + DES$_0$ group than in the saline group ($P < 0.01$), whereas the number of TH-positive neurons in the substantial nigra was slightly increased in the MPTP + DES$_1$ group compared with that in the MPTP + DES$_0$ group ($P < 0.05$). The density of TH-positive dopaminergic fibers in the striatum in the MPTP + DES$_0$ group was reduced compared with that in the saline group but without statistical significance (Figure 3c).
Iba1 and GFAP levels in MPTP-induced PD mice were suppressed by transient DES inhalation (1 hour) in the substantial nigra, striatum, and hippocampus

Iba1 positivity represents the activation of microglial cells in which it is highly and specifically expressed. As an astrocytic marker, GFAP can be measured to represent the activation of astrocytes. As shown in Figure 4a, b, and c, the fluorescence intensities of Iba1 and GFAP were significantly increased in the MPTP + DES0 group compared with those in the saline group \((P < 0.01)\) but decreased in the MPTP + DES1 group compared with those in the MPTP + DES0 group \((P < 0.01)\) in the substantial nigra, striatum, and hippocampus. The fluorescence intensities of Iba1 and GFAP from images were quantified and shown in Figure 4 (a2, a3, b2, b3, c2, and c3).

Increases in inflammation-associated proteins (p-p38, TLR4, and TNF-\(\alpha\)) in the hippocampus from MPTP-induced PD mice were inhibited by transient DES inhalation (1 hour)

As shown in Figure 5, the protein levels of p-p38, TLR4, and TNF-\(\alpha\) examined by western blotting were higher in the
MPTP + DES₀ group than in the saline group in the hippocampus ($P < 0.05$) but lower in the MPTP + DES₁ group than in the MPTP + DES₀ group ($P < 0.05$).

**Discussion**

In the present study, we successfully replicated a chronic PD model in mice through...
Figure 4. Effects of DES on Iba1 and GFAP levels in PD model mice. The levels of Iba1 and GFAP were suppressed by DES inhalation for 1 hour. Iba1 and GFAP were stained with green in separate images, and nuclei were stained with DAPI (blue) (a1, b1, and c1: magnification of 60 ×, scale: 20 μm). The quantified fluorescence intensities of Iba1 (a2, b2, and c2) and GFAP (a3, b3, and c3) positive cells per high power field and (d) A high magnification of Iba1 and GFAP was illustrated (magnification of 80 ×, scale: 10 μm). *P < 0.05 and **P < 0.01 vs the control group, #P < 0.05 and ###P < 0.01 vs the MPTP + DES0 group.

DES, desflurane; Iba1, ionized calcium binding adaptor molecule-1; GFAP, glial fibrillary acid protein; PD, Parkinson’s disease; MPTP, 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine; DAPI, 4′,6-diamidino-2-phenylindole.
intraperitoneal daily injections with MPTP for 20 consecutive days, and this PD mice model was validated with the pole, hanging-wire, and open field tests and the loss of dopaminergic neurons with TH-positive staining in the substantia nigra. Interestingly, our results first demonstrated the neuroprotective effects of DES on MPTP-induced pathogenesis in PD mice, and only an appropriate administration regimen of DES (1 hour inhalation of a mixture of 7.5% DES + 100% oxygen) provided these effects, longer DES inhalations of 2 or 4 hours did not. The neuromuscular strength of MPTP-induced PD mice hanging on the wire and motor behavior deficits were greatly ameliorated, although the degree of recovery did not return to the normal level. The loss of TH-positive neurons in the substantia nigra was due to
MPTP, but the transient inhalation of DES (1 hour) alleviated the detrimental damage induced by MPTP. However, differences in the TH-positive fiber density in the striatum between the MPTP model group and saline group were not statistically significant. The alterations in the function and density of dopaminergic neurons in the substantia nigra of mice induced by MPTP were different from those in the striatum.\textsuperscript{7,23}

The nigrostriatal pathway originates from the substantia nigra and terminates within the striatum, and the selective depletion of dopamine in the striatum pathway is less susceptible to MPTP.\textsuperscript{24,25}

In the present study, microglial cells and astrocytes were activated by MPTP, as reflected by the immunostaining of Iba1 and GFAP. Nevertheless, the activation of these microglial cells and astrocytes was
inhibited by the transient inhalation of DES. To gain insight into the inflammatory role of MPTP in the PD mouse model, associated molecules or mediators, including p-p38, TLR4, and TNF-α, in the hippocampus were examined by western blotting. The results suggested that their levels were upregulated by MPTP injection. However, these increases in p-p38, TLR4, and TNF-α induced by MPTP were inhibited by the transient inhalation of DES (1 hour).

**Figure 4.** Continued.

**Figure 5.** Effects of DES on inflammatory mediators. The increases in inflammatory proteins were inhibited by DES inhalation for 1 hour. Protein levels (p38, p-p38, TLR4, and TNF-α) using western blotting. Representative images of samples from the hippocampus in the saline group and PD groups treated with DES. Colorimetric densities of individual bands were quantitatively measured. Actin was used as a control.

*P < 0.05 vs the control group, #P < 0.05 vs the MPTP + DES0 group.

DES, desflurane; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor-alpha; PD, Parkinson’s disease; MPTP, 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine.
PD is an irreversible neurodegenerative disease resulting from the progressive degeneration of the substantia nigra and disruption of the dopaminergic nigrostriatal pathway. It is widely accepted that the MPTP-induced pathogenesis in the mouse PD model more likely occurs in the substantia nigra than in the striatum. The MPTP metabolite 1-methyl-4-phenylpyridinium greatly contributes to the damaged dopaminergic neurons in the substantia nigra, activated glial cells, and reduced microtubule-associated protein 2 levels. TLR4 also plays an important role in the pathogenesis because it was found to be highly expressed on the microglial cell membrane and upregulated in the MPTP-induced mouse PD model. Our present results indicated that both microglial cells and astrocytes were activated in the PD mouse model induced by MPTP. Together with released cytokines, several inflammatory mediators were produced by microglial cells or astrocytes, as evidenced by the upregulation of p-p38, TLR4, and TNF-α in the hippocampus. Nevertheless, an anti-inflammatory effect of DES inhalation is plausible because different experimental designs and conditions with various administration regimens of DES inhalation have provided controversial results, and it is difficult to draw a consistent conclusion. A longer 4 hour inhalation of DES at a 1.0 minimum alveolar concentration in the swine model increased oxidative stress and reactive oxygen species products, and DES inhalation for shorter than 1 hour was unable to provide sufficient protection. DES at a 0.5 mM concentration in vitro inhibited H₂O₂ production. Moreover, DES provided more cardio-protection than propofol.

The present results showed that post-treatment with 7.5% DES for 1 hour inhibits the inflammatory response in the central nervous system induced by MPTP, indicating that the anti-inflammatory effect of DES in the central nervous system is dependent on the duration of inhalation. However, the detailed underlying mechanism requires further study. According to our results, we speculate that the following mechanisms are feasible. With appropriate exposure to DES, its anti-inflammatory and/anti-oxidation effects are greater than its oxidative stress activity, whereas with prolonged exposure to DES, its oxidative stress activity is greater than its antioxidant effect. Inhalation for 1 hour can only reduce but not eliminate the inflammation in the central nervous system induced by MPTP. Inhalation for 2 and 4 hours cannot reduce neuroinflammation, but it does not increase inflammation. Therefore, we believe that DES, as an anesthetic drug rather than a therapeutic drug, has no reversal effect on the course of PD, and no conclusion can be drawn regarding the effect of DES on neuroinflammation in PD mice. The limitation of this study is that it did not observe whether DES inhalation at different concentrations for 1 hour still provides the above-mentioned neuroprotective effect on PD mice induced by MPTP.

**Conclusion**

Our study demonstrates that the transient inhalation of DES for 1 hour but not 2 or 4 hours ameliorates locomotor dysfunction and alleviates neuroinflammation in the brain by disrupting the cross-talk between microglial cells and astrocytes and inhibiting the p38/TLR4 inflammatory pathway. Accordingly, DES not only serves as a safer anesthetic for patients with PD but also provides potential protection against PD.

**Declaration of conflicting interest**

The authors declare that there is no conflict of interest.
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