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Gintonin Mitigates MPTP-Induced Loss of Nigrostriatal Dopaminergic Neurons and Accumulation of α-Synuclein via the Nrf2/HO-1 Pathway

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Abstract

Gintonin, a ginseng-derived glycolipoprotein isolated from ginseng, has been shown to be neuroprotective in several neurological disorders such as Alzheimer's disease models and depressive-like behaviors. In this study, we sought to investigate the potential protective mechanisms of gintonin in an in vivo MPTP and in vitro MPP^+-mediated Parkinson's disease (PD) model. We hypothesized that activation of nuclear factor erythroid 2-related factor 2/heme oxygenase-1 (Nrf2/HO-1, potential therapeutic targets for neurodegeneration) with gintonin could abrogate PD-associated neurotoxicity by modulating the accumulation of α-synuclein, neuroinflammation, and apoptotic cell death in an MPTP/MPP^+ models of PD. Our in vivo and in vitro findings suggest that the neuroprotective effects of gintonin were associated with the regulation of the Nrf2/HO-1 pathway, which regulated the expression of proinflammatory cytokines and nitric oxide synthase and apoptotic markers in the substantia nigra and striatum of the mice. Moreover, the neuroprotective effects of gintonin were also associated with a reduction in α-synuclein accumulation in the mouse substantia nigra and striatum. The neuroprotective effects of gintonin were further validated by analyzing the effects of gintonin on MPP^+-treated SH-SY5Y cells, which confirmed the protective effects of gintonin. It remains for future basic and clinical research to determine the potential use of gintonin in Parkinson's disease. However, to the best of our knowledge, marked alterations in biochemical and morphological setup of midbrain dopaminergic pathways by gintonin in MPTP mice model have not been previously reported. We believe that gintonin might be explored as an important therapeutic agent in the treatment of PD.

Keywords Gintonin · Neuroprotection · Nrf2/HO-1 pathway · Parkinson's disease · MPTP · Neuroinflammation

Introduction

Parkinson's disease (PD) is a progressively neurodegenerative disorder that affects approximately 0.3% of the entire world population [1–3]. It is characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and depletion of dopamine in the striatum, as well as by the presence of intracytoplasmic inclusions of aggregated proteins designated by Lewy bodies [4, 5]. Although the leading cause of dopaminergic cell loss remains elusive, the loss seems to be associated with interconnected cell damage mechanisms, particularly mitochondrial dysfunction and oxidative stress [4, 6–8].

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PD pathology includes neuronal loss in specific regions as well as accumulation of α-synuclein at intracellular spaces [9]. Early stage of PD comprises loss of dopaminergic neurons in ventrolateral substantia nigra, but widespread pathology is evident at late stages which leads to onset of motor symptoms [10]. Abnormal cytoplasmic deposition of α-synuclein in neurons of different brain region leads to aggregation of Lewy bodies. Lewy body aggregation starts from cholinergic and nondopaminergic neurons in olfactory system but with disease progression also detected in neocortical and limbic region of brain [11]. Among all PD cases, only 5–10% are heritable. Genome-wide association studies confirm genes involved in onset of sporadic PD involved in different molecular pathways like α-synuclein proteostasis, oxidative stress, mitochondrial dysfunction, disruption in calcium homeostasis, impaired axonal transport, and neuroinflammation [12].

Among all clinical symptoms, aggregation of α-synuclein is found in all PD patients. Mutations in SNCA gene which encode α-synuclein protein are a major contributor of PD [13]. Normal function of 140 amino acid cytoplasmic neuronal protein is still not completely known. The presence of mutations in encoding gene is supposed to be responsible for misfolding, oligomerization, and decline in proteolytic degradation of aggregated protein [14]. Progression in age plays a major role in that decline in proteolytic defense mechanism causes severity in symptoms.

Mitochondrial dysfunction is regarded as one of the key pathological factors in disease progression. Several scientific evidences suggest that mitochondrial dysfunction and α-synuclein affect each other and exacerbate disease symptoms [15]. Recent studies suggest that several genes including LRRK2, PARK2, and PINK1 are responsible fot the mitochondrial impairment along with MIRO which plays a role in clearance of damages in mitochondria and cause energy deficit [16].

Oxidative stress is a consequence of mitochondrial impairment. Among all organs, the brain is the most sensitive and susceptible to oxidative stress. ROS is generated by a number of pathways, but the initial free radical reactions require activation of molecular oxygen [17]. ROS is continuously generated in vivo as a result of oxygen metabolism; approximately 1–5% of the oxygen consumed is converted to ROS [18]. The brain is considered the most susceptible organ to oxidative stress and damage, as the brain consumes more oxygen than any other organ and contains relatively low levels of the antioxidant system compared to other tissues, as well as high amounts of phospholipids, which are highly vulnerable to oxidative changes [19, 20]. Consequently, neuronal cell loss may occur due to an imbalance between mitochondrial ROS and endogenous ROS scavengers [21, 22].

To maintain a proper physiological redox balance, cells are endowed with a wide range of endogenous antioxidant enzymes [23–25]. Upon ROS exposure, several of these enzymes are activated by the transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2), the master regulator of cellular redox status [26, 27]. Once activated, Nrf2 regulates different Nrf2 downstream targets, the most important being heme-oxygenase (HO) [28]. Accordingly, overexpression of these proteins under neurotoxic conditions prevents hydrogen peroxide accumulation, lipid peroxidation, and, consequently, neuronal loss [29].

Ginseng has been used in the prehistorically traditional herbal vital medicine along with various physiological and pharmacological effects on nervous and other body systems, brain homeostasis, and cognitive performance in human and AD patients [30, 31]. Evidences showed in recent studies that ginseng extracts increased hippocampal neurogenesis after focal cerebral ischemia [32]. However, the active ingredient of ginseng and signaling mechanisms have known not much. Gintonin, a form of herbal medicine GTP-coupled lysophosphatidic acid, was isolated glycolipoprotein from ginseng [33]. Gintonin was recently identified as an active ingredient of ginseng, the root of *Panax ginseng* in C.A. Meyer [34]. Among several ingredients isolated from ginseng, gintonin is a very valuable compound. It is a glycolipoprotein that has a complex of lysophosphatidic acids (LPA) and major latex like protein 151 [35]. In several lines of evidence, gintonin upregulated synaptic transmission in hippocampal regions [34, 36]. Gintonin activated the non-amyloidogenic pathways and enhanced long-term memory impairment in Alzheimer's disease transgenic animal model, attenuated amyloid beta plaque deposition, and downregulated glial activation in hippocampus [37]. Also, it has been shown to improve performance of mice in a rotarod test [38], attenuate depressive-like behaviors associated with alcohol withdrawal in mice [39], and stimulate gliotransmitter release in cortical primary astrocytes [40].

Based on the previously defined beneficial effects of ginseng, here, we hypothesized that the natural compound gintonin may exert a protective effect on dopaminergic neuronal cells both in vitro and in vivo. Here, we noted that gintonin activates the Nrf2/HO-1 signaling pathway, induces antioxidant defense enzymes, regulates inflammatory mediators, confers neuronal survival, and normalizes motor deficits in an animal model of PD. Subsequently, we demonstrated that Nrf2 may be a promising target for gintonin to reduce ROS-mediated damage in PD, potentially leading to interesting therapeutic approaches.

Materials and Methods

Chemicals and Antibodies

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (MPTP) was obtained from Sigma-Aldrich (St. Louis, MO,

USA). Gintonin was kindly provided by Professor Hyewhon Rhim. Gintonin was prepared from Panax-ginseng without saponin according to described methods previously [33]. The antibody against tyrosine hydroxylase (TH) was obtained from Millipore (Merck Millipore, MA, USA). Antibodies against caspase-3, Nrf2, HO-1, Bcl-2, Bax, COX-2, NOS-2, TNF-α, glial fibrillary acidic protein (GFAP), ionized calcium-binding adapter molecule-1 (Iba-1), and β-actin were obtained from Santa Cruz Biotechnology (Dallas, TX, USA).

Animal Groupings and Drug Administration

Male C57BL/6N wild-type (WT) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The mice were kept in the university animal house under a 12/12-h light and dark cycle at room temperature, 60 ± 10% humidity with food and water ad libitum. After a 1-week acclimatization, mice were randomly divided into four groups ($n = 10$ mice/group). The MPTP group was injected with MPTP at a dose of 30 mg/kg i.p. for 5 days, as used previously [6, 41], and the control group was administered an equal volume of saline (0.9% NaCl). Gintonin was dissolved in vehicle saline solution before use for i.p. injection [36, 42]. We used the similar mean effective dose concentrations, and gintonin was used in two doses: one group was given MPTP + gintonin 50 mg/kg and another MPTP + gintonin 100 mg/kg [33, 42, 43]. After the completion of the 5-day MPTP treatment, the mice were allowed to recover. Then, gintonin was administered with an oral injector for 21 days at a dose of 50 and 100 mg/kg. For the handling and MPTP administration, the previously described protocols were followed [44].

Extraction of the Protein from Mouse Brain

After completion of the treatment and behavior analysis, the mice were sacrificed, and the brain tissue (striatum and substantia nigra) was carefully removed. The tissue was frozen on dry ice and stored at − 70 °C. Then, the brain tissue was homogenized in Pro-Prep TM protein extraction solution according to the provided instructions (iNtRON Biotechnology, Sungnam, Republic of Korea). The samples were centrifuged at 13,000 rpm at 4 °C for 25 min. The supernatants were collected and stored at − 70 °C for further analysis and assays.

Cell Culture and Transfection

SH-SY5Y human neuroblastoma cells (Korea Cell Line Bank) were cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogen, CA, USA) with 10% FBS. The EGFP-alphasynuclein-A53T plasmid which is from David Rubinsztein (Addgene, Cambridge, MA, plasmid #40823) was used. According to the manufacturers, these cells were transfected with EGFP-alphasynuclein-A53T plasmid and Lipofectamine 3000 (Invitrogen, CA, USA).

Western Blot Analysis

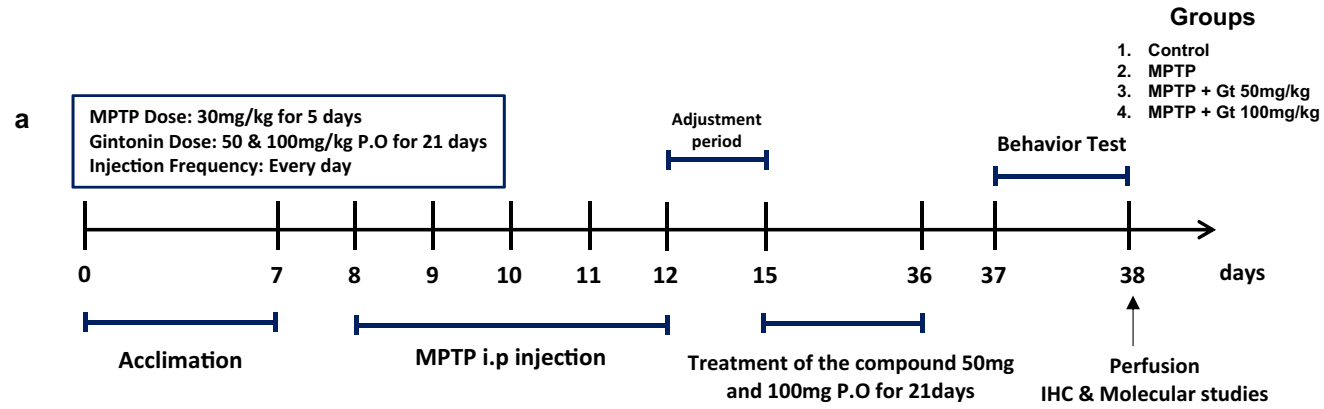
Western blot analysis was performed according to previously reported methods with little modifications [45–47]. Briefly, the protein concentrations were analyzed with a Bio-Rad protein assay kit (Bio-Rad Laboratories, CA, USA). Equal amounts of proteins (20 μg) were subjected to electrophoresis on 10–20% Bolt™ Mini Gels and separated on 0.2-μm PVDF Immobilon membranes (Merck Millipore, MA, USA). To reduce nonspecific binding, the membranes were blocked in 5% (*w*/*v*) skim milk and then incubated with the primary antibodies (1:1000 dilution) overnight at 4 °C. After the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies, the specific bands were detected by EzWestLumiOne (ATTO, Tokyo, Japan) in a dark room as performed previously with slight modifications [48]. The Super RX-N X-ray films (Fujifilm, Tokyo, Japan) were scanned, and the optical densities of the bands were analyzed by densitometry using the computer-based ImageJ software (NIH, MA, USA).

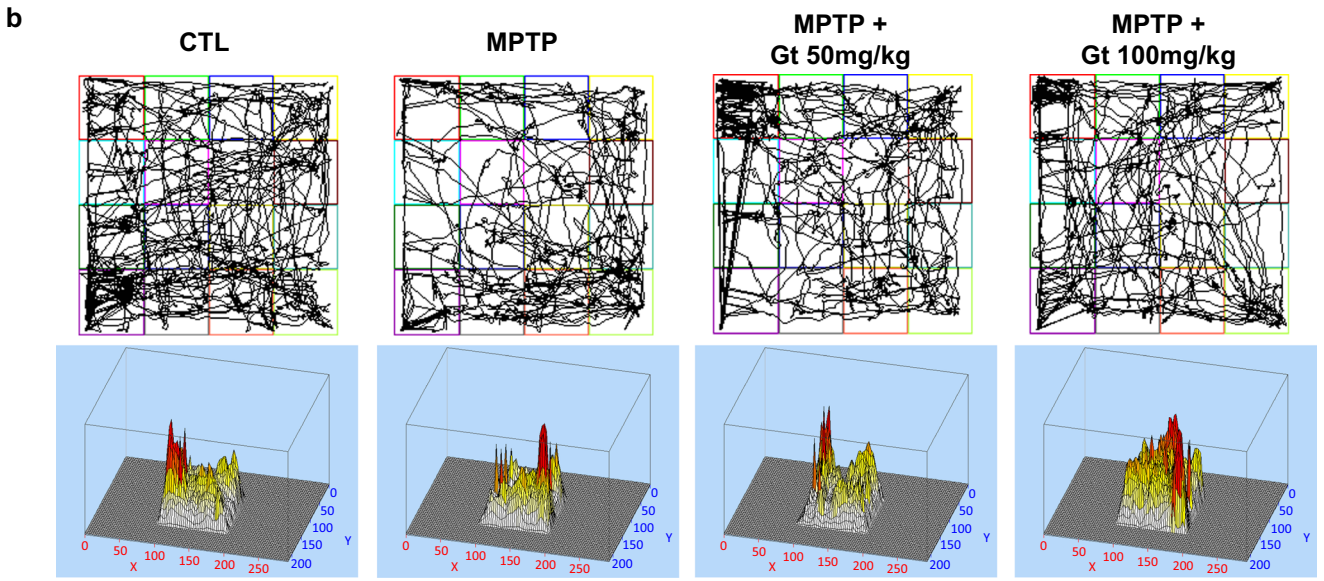
ROS Assay In Vivo

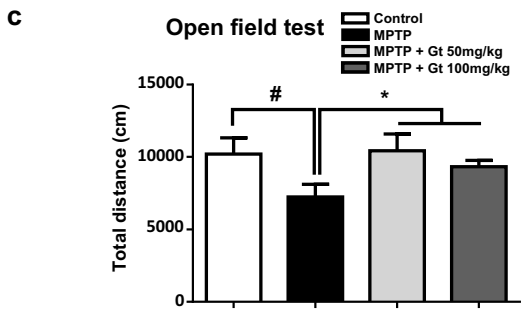
A ROS assay was performed as described previously [47]. The ROS assay is based on the oxidation of 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) to 2′,7′-dichlorofluorescein (DCF). The brain homogenates of the substantia nigra and striatum of the different groups were diluted with ice-cold Lock's buffer at a 1:20 ratio to yield the final concentration of 2.5 mg tissue/500 μl. The reaction mixture containing Lock's buffer (1 ml, pH ± 7.4), 0.2 ml of homogenate, and 10 ml of DCFH-DA (5 mM) was incubated at room temperature for 15 min to convert DCFH-DA to the fluorescent product DCF. The conversion of DCFH-DA to DCF was evaluated using a spectrofluorometer (Promega BioSciences, CA, USA) with excitation at 484 nm and emission at 530 nm. For background fluorescence (conversion of DCFH-DA in the absence of homogenate), we measured parallel blanks. The values were expressed as DCF formed pmol/protein mg.

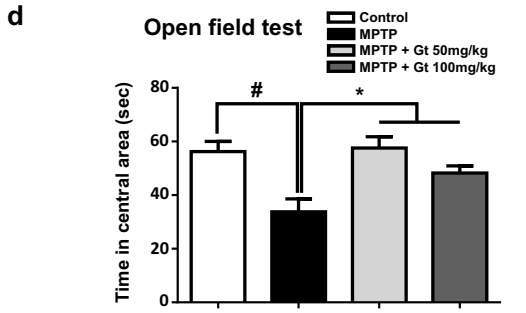
Tyrosine Hydroxylase Immunohistostaining

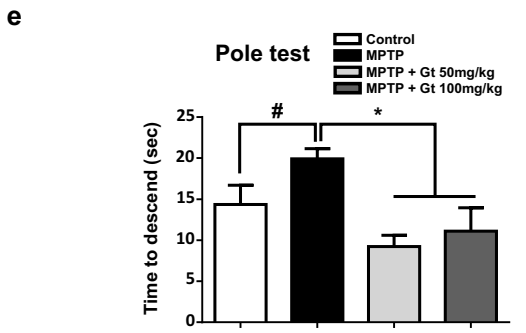
Tyrosine hydroxylase (TH) immunostaining was performed to analyze the number of TH neurons in the SNpc. For that purpose, the slides containing the sections were washed with PBS (0.01 M) for 5 min, treated with 30% H_2O_2 in methanol for 10 min, and then treated with normal goat serum (5%) containing 0.1% Triton X-100 in PBS for 90 min. The slides were treated with the TH antibody (Merck Millipore, MA,

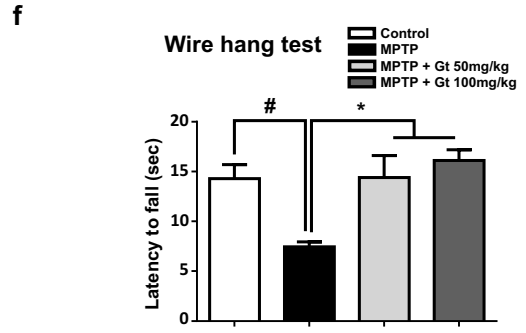

Groups
1. Control
2. MPTP
3. MPTP + Gt 50mg/kg
4. MPTP + Gt 100mg/kg
a
MPTP Dose: 30mg/kg for 5 days
Gintonin Dose: 50 & 100mg/kg P.O for 21 days
Injection Frequency: Every day
Adjustment period
Behavior Test
0 7 8 9 10 11 12 15 36 37 38 days
Acclimation
MPTP i.p injection
Treatment of the compound 50mg and 100mg P.O for 21days
Perfusion
IHC & Molecular studies


b
CTL
MPTP
MPTP + Gt 50mg/kg
MPTP + Gt 100mg/kg


c
Open field test
Control
MPTP
MPTP + Gt 50mg/kg
MPTP + Gt 100mg/kg
Total distance (cm)
15000
10000
5000
0
#
*


d
Open field test
Control
MPTP
MPTP + Gt 50mg/kg
MPTP + Gt 100mg/kg
Time in central area (sec)
80
60
40
20
0
#
*


e
Pole test
Control
MPTP
MPTP + Gt 50mg/kg
MPTP + Gt 100mg/kg
Time to descend (sec)
25
20
15
10
5
0
#
*


f
Wire hang test
Control
MPTP
MPTP + Gt 50mg/kg
MPTP + Gt 100mg/kg
Latency to fall (sec)
20
15
10
5
0
#
*

◀ **Fig. 1** Gintonin abrogated motor dysfunction in MPTP-induced PD model. **a** In vivo scheme of study. **b** Representative images of the mouse movements in the open field box, as captured by the video tracking system. **c** Quantitative analysis of the total distance covered by the mice in the open field box. **d** Representative histogram showing the effects of gintonin on MPTP-induced anxiety-like behaviors. **e** Representative histogram of the pole test results, showing an increase in the total time to descend to the floor in the MPTP-treated group compared to that in the vehicle-treated control group in the pole test, which was reduced with gintonin treatment. **f** Histogram showing the results of the wire hang test, according to which gintonin significantly enhanced the hanging time compared to MPTP treatment alone ($n = 10$ mice/group). #Significantly different from the control group; *significantly different from MPTP-treated mice. Significance: $^{*}P < 0.05$; $^{\#}P < 0.05$

USA) as a primary antibody for 16 h in normal goat serum (5%) as a blocking solution at 4 °C. Next, the slides were treated with a secondary goat anti-rabbit IgG antibody from Santa Cruz Biotechnology (Dallas, TX, USA) at room temperature for 120 min, incubated in ABC reagent (Vector Laboratories, CA, USA) at room temperature for 1 h, and developed with 3,3-diaminobenzidine tetrahydrochloride hydrate (DAB) solution (Sigma-Aldrich, St. Louis, MO, USA) with 30% H_2O_2. Finally, the slides were dehydrated in different percentages of ethanol (70 and 95%), dipped in xylene, and covered with a thin cover glass by using DPX neutral mounting medium (Sigma-Aldrich, St. Louis, MO, USA). The samples were inspected by an Olympus AX70 microscope (Olympus, Tokyo, Japan).

Determination of Lipid Peroxidation

Lipid peroxidation (LPO) is another assay used to evaluate oxidative stress. For this purpose, free malondialdehyde (MDA) (a marker of LPO) was measured in the homogenates of the substantia nigra and striatum of the different experimental groups using a lipid peroxidation colorimetric/fluorometric assay kit (BioVision, CA, USA, Cat# K739-100) according to the protocols provided by the manufacturer and previously performed by our group [47].

Cresyl Violet (Nissl) Staining

Cresyl violet (nissl) staining was performed in accordance with modified protocols [49]. Slide samples were overnight dried at room temperature and washed in 0.01 M PBS twice. It was stained using 0.1% cresyl violet solution (Sigma-Aldrich, St. Louis, MO, USA) for 15 min. The brain slides were dehydrated by 70% ethanol and absolute ethanol. Then, it was treated by xylene for clearing tissues and mounted by DPX mounting medium (Sigma-Aldrich, St. Louis, MO, USA). The sample images were inspected using an Olympus AX70 microscope (Olympus, Tokyo, Japan).

Immunofluorescence

After completion of the treatments and behavioral analysis, the mice ($n = 5$ per group) were deeply anesthetized and perfused with normal saline solution (0.9%) and 4% cold paraformaldehyde. After transfusion, the brain samples were removed and placed in paraformaldehyde solution for 48 h at 4 °C, followed by immersion in 30% sucrose for the next 72 h. To ensure optimal cutting temperature, the brain tissues were fixed in OCT compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan), and the midbrain samples containing the SNpc were serially cut into 14-μm-thick coronal sections using a cryomicrotome (Leica cryostat CM 3050, Germany). The slides containing the brain samples were dried at room temperature for 24 h, next day washed with PBS (0.01 mM) for 8–10 min two times. Then, the slides were treated with proteinase K for 5 min, rinsed with PBS (0.01 mM), and blocked with 2% goat normal serum in PBS containing 0.1% Triton X-100 for 50 min. The slides were incubated with primary antibodies overnight at 4 °C including anti-GFAP (Santa Cruz Biotechnology, Dallas, TX, USA) and α-synuclein (Cell Signaling Technology, Beverly, MA, USA). After incubation with primary antibodies for 24 h, the slides were incubated with FITC or TRITC-labeled secondary antibodies in PBS (anti-rabbit or anti-mouse) for 90 min at room temperature. Slides were washed twice with PBS for 5 min. By using fluorescent mounting medium (Dako 53023), glass coverslips were mounted on slides. Images were taken using a confocal laser-scanning microscope (FluoView FV 1000 MPE, Olympus, Tokyo, Japan).

Open Field Test

The spontaneous locomotor activity was evaluated by using an open field system (40 × 40 cm in diameter, 40 cm in height) [50], as used previously with slight modification [41, 51]. The open field box was divided into 16 equally sized squares, and the movements of the mice were recorded by using a video tracking software 3.0 (Panlab, Barcelona, Spain). The mice were allowed to adjust to the new environment for a few hours before the experiment. After that, the mice were individually placed in the same corner. The experiments were conducted in a calm and dimly lit room to prevent distractions and accidentally cause freezing behavior [52]. Between each test, the open field was scrubbed with ethanol solution (10%) and dried to remove odor. The total distance traveled by the mice and the time spent in the central area were evaluated. The distance is presented in centimeters, while the time is presented in seconds.

Pole Test

The behavioral tests were performed after the completion of the treatment. The pole test was performed by using the previously used method with slight

modifications [53]. Mice were positioned head up near the top of a rough-surfaced wooden pole (10 mm in diameter and 40 cm in height), and the time taken to reach the floor was considered. The test was repeated three times, and behavioral alterations were analyzed according to the mean of the three descending times.

Wire Hang Test

The neuromuscular strength was determined by the wire hang test, also called the traction test [6]. Before the test, the mice were acclimated to the behavioral room for 20–30 min. Mice were raised by their tail, placed smoothly on a horizontally stretched wire, and supported until they grabbed the wire with both of their hind and fore paws as described previously with slight modifications. The wire was mounted 20 cm above the ground surface to discourage falling but not cause any sort of injury in case of a fall, [54]. The experimental procedures were reiterated 10 times, and the average values were used in the evaluation. The mice were allowed to rest between the trials. The results are described in seconds, as a latency to fall to the ground.

Statistical Analysis

The X-ray films were scanned and the expression of the bands were analyzed via densitometry using the computer-based ImageJ program. The density values are expressed as the mean ± standard error of the mean (SEM). A one-way ANOVA (analysis of variance) followed by independent Student's *t* test and Tukey's multiple comparison analysis were used for comparison among the different groups. The graphs were generated with Prism 6 software (GraphPad Software, Inc., San Diego, CA, USA). *P* values < 0.05 were taken to be statistically considerable: #significantly different from the control group, *significantly different from the MPTP-treated groups. $*P < 0.05$ and $\#P < 0.05$. (Control, treated with saline; Gt, treated with oral gintonin; MPTP, treated with MPTP; MPTP + Gt, injected with MPTP and orally administered gintonin).

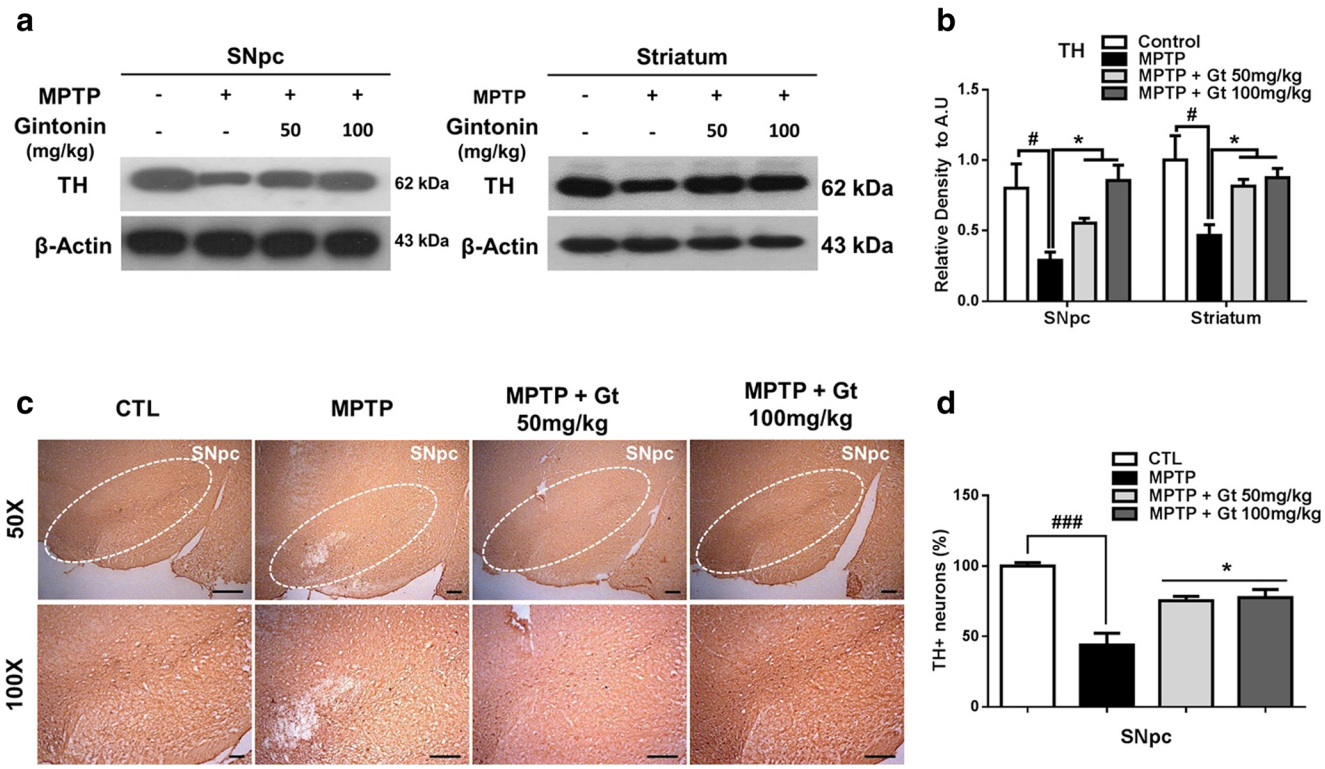


Fig. 2 Gintonin upregulated the TH level in the substantia nigra pars compacta (SNpc) and striatum of the mouse brain. **a** Images of the scanned western blot results showing TH expression in the substantia nigra and striatum of different experimental animals. **b** Representative histograms showing the effects of gintonin on the MPTP-induced TH level in the substantia nigra (SNpc) and striatum of the mice. **c**, **d** Images of the immunostaining results and the corresponding histogram. The values are expressed as the mean ± SD for indicated proteins ($n = 10$ mice/group), and the number of independent experiments was $n = 3$. #Significantly different from the control group; *significantly different from the MPTP-treated group. Significance: $*P < 0.05$; $\#P < 0.05$; $\#\#\#P < 0.001$

Results

Gintonin Abrogated Motor Dysfunction in the MPTP-Induced PD Model

The MPTP-induced PD model is a well-known model used to explore the molecular alterations related to Parkinson's disease, which causes motor dysfunction and behavioral alterations, as already mentioned in different studies [55, 56]. To address the effects of gintonin on motor dysfunction and behavioral alterations, we performed some basic behavioral studies, such as an open field test, pole test, and wire hang test. As shown in Fig. 1c, MPTP treatment significantly reduced the total distance covered by the mice in the open field box compared to saline i.p. injection, an effect that was reversed with the treatment of gintonin at both 50 and 100 mg/kg. Similarly, the time spent in the central area was reduced with MPTP treatment and was significantly upregulated with the treatment of gintonin, as shown in Fig. 1d. In the pole test, the total time to descend to the floor was significantly increased with MPTP treatment compared to that in the control group, as previously reported [51]. With 50 and 100 mg/kg gintonin treatment, the latency to reach the floor was significantly reduced in both cases, as shown in Fig. 1e. In the wire hang test, MPTP reduced the hanging time, indicating

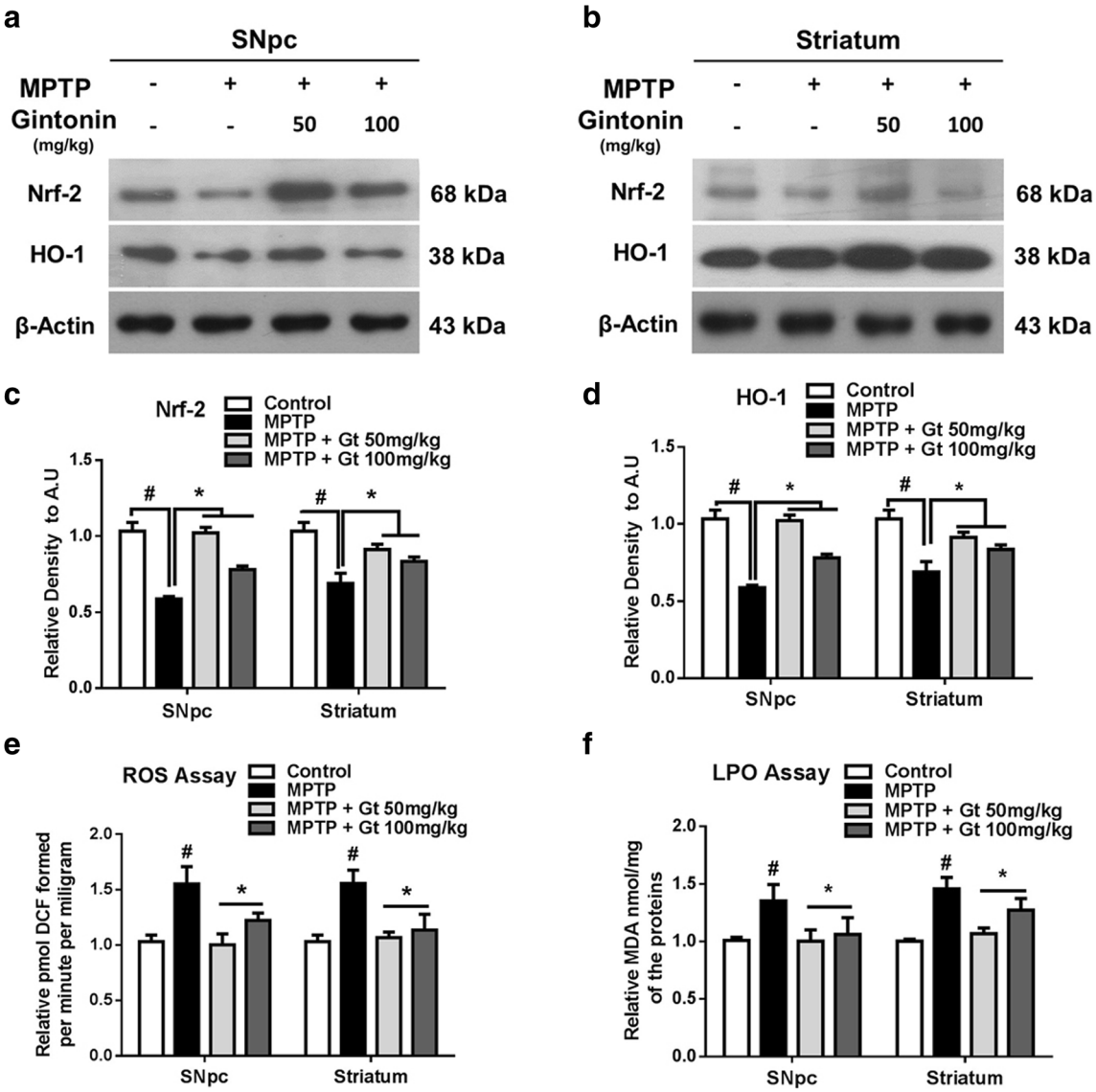


Fig. 3 Gintonin regulated the expression of the Nrf2/HO-1 pathway in the substantia nigra and striatum of mice. **a**, **b** Images of the scanned immunoblot results of Nrf2 and HO-1 in the substantia nigra and striatum of the different experimental groups. **c**, **d** Graphical representation of the expression of Nrf2/HO-1 in the substantia nigra and striatum of the different experimental groups. **e** Graphical representation of the ROS level in the substantia nigra and striatum of the different experimental groups. **f** Graphical representation of the results of the lipid peroxidation assay in the different experimental groups ($n = 10$ mice/group). #Significantly different from the control group; *significantly different from MPTP-treated mice. Significance: $^{*}P < 0.05$; $^{\#}P < 0.05$

that the neuromuscular strength was significantly compromised by MPTP treatment, as mentioned previously [57]. This effect was abrogated with gintonin treatment at 50 and 100 mg/kg, as shown in Fig. 1f.

Gintonin Protected Dopaminergic Neurons by Preserving the TH Level in the Substantia Nigra and Striatum

Tyrosine hydroxylase (TH) is a key regulator for the maintenance and regulation of mesencephalic neurons and is reduced in PD patients [58]. The level of TH has been reported to be decreased in MPTP-injected (i.p.) mice compared to that in control mice [44, 59]; therefore, we evaluated the effects of gintonin on TH expression in MPTP-treated mice. According to our results, gintonin at 50 and 100 mg/kg significantly upregulated the decreased level of TH in the substantia nigra and striatum, as shown in Fig. 2a. We confirmed the beneficial effects of gintonin on the MPTP-induced TH loss in the substantia nigra by performing TH immunostaining, which affirmed the hypothesis that gintonin restored the reduced expression of TH in MPTP-treated mice, as shown in Fig. 2c, d.

Gintonin Protected the Substantia Nigra and Striatum of the Mouse Brain Against MPTP-Induced Oxidative Stress via the Nrf2/HO-1 Signaling Pathway

Oxidative stress is a main contributor to the pathogenesis of Parkinson's disease [60]. Previously, Nrf2 has been shown to act as an antioxidant defense mechanism in protecting the cells against abnormal ROS levels. The nuclear translocation of Nrf2 and the expression of its target gene HO-1 elicit an antioxidant defense system that is considered to guard the dopaminergic cells against MPTP-induced oxidative stress [61]. Therefore, we assessed the effects of gintonin on the activation of the Nrf2/HO-1 pathway in the brain homogenates of MPTP-treated mice. According to our western blot results, MPTP reduced the expression of Nrf2 and HO-1 in the substantia nigra and striatum of the mouse brain compared to that in the control animals, and the expression levels were upregulated by gintonin treatment, as

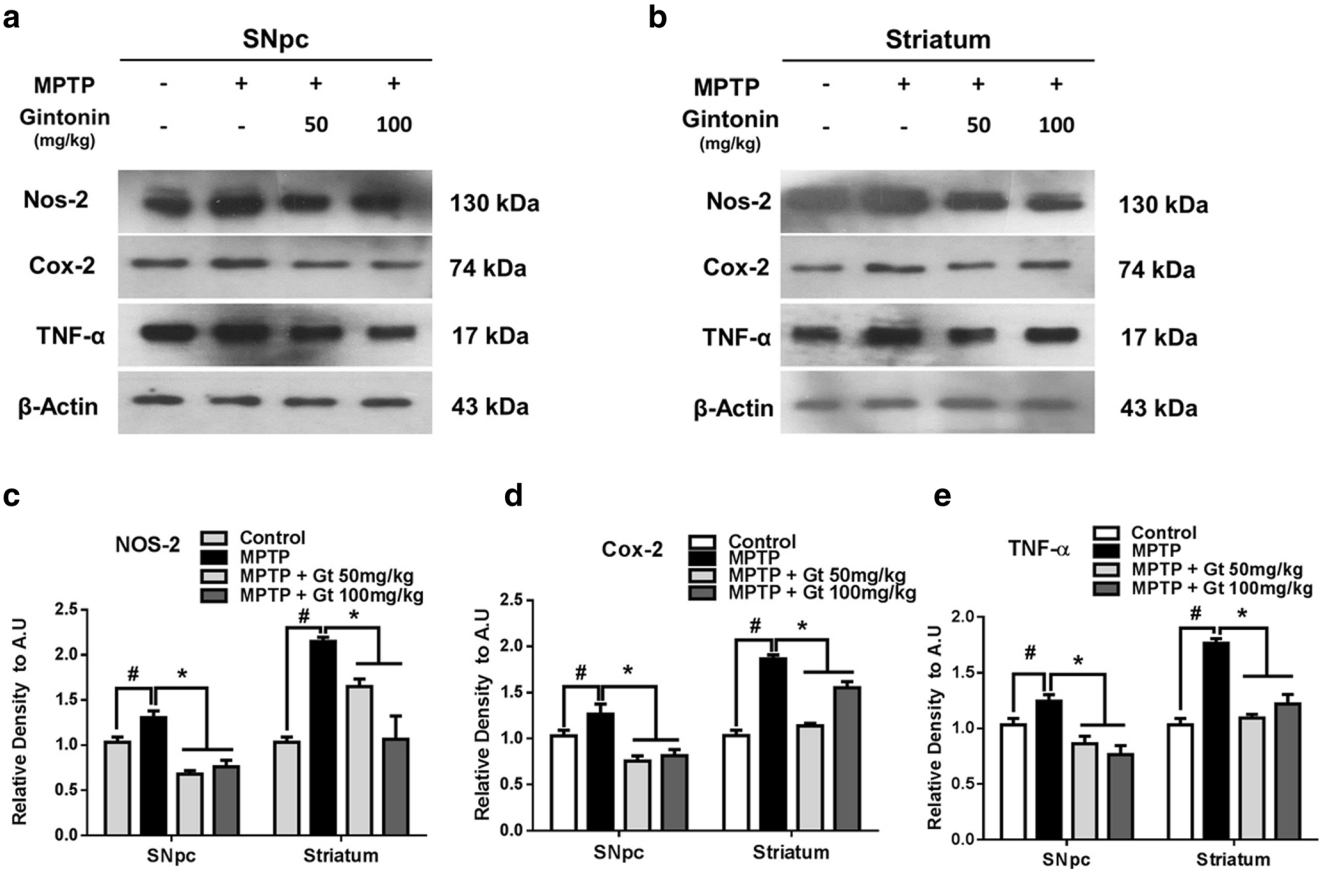


Fig. 4 Gintonin regulated the expression of inflammatory cytokines in substantia nigra and striatum of mice. **a**, **b** Images of the scanned immunoblot results for NOS-2, COX-2, and TNF in the substantia nigra and striatum of the mice in the different groups tested. **c, d, e** Quantitative analysis of the effects of MPTP and gintonin on the expression of the inflammatory cytokines (NOS-2, COX-2, and TNF-α) in the substantia nigra and striatum of the mice. The values are expressed as the mean ± SD for indicated proteins, and the number of independent experiments was $n = 10$ mice/group. #Significantly different from the control group; *significantly different from MPTP-treated mice. Significance: $^{*}P < 0.05$; $^{\#}P < 0.05$

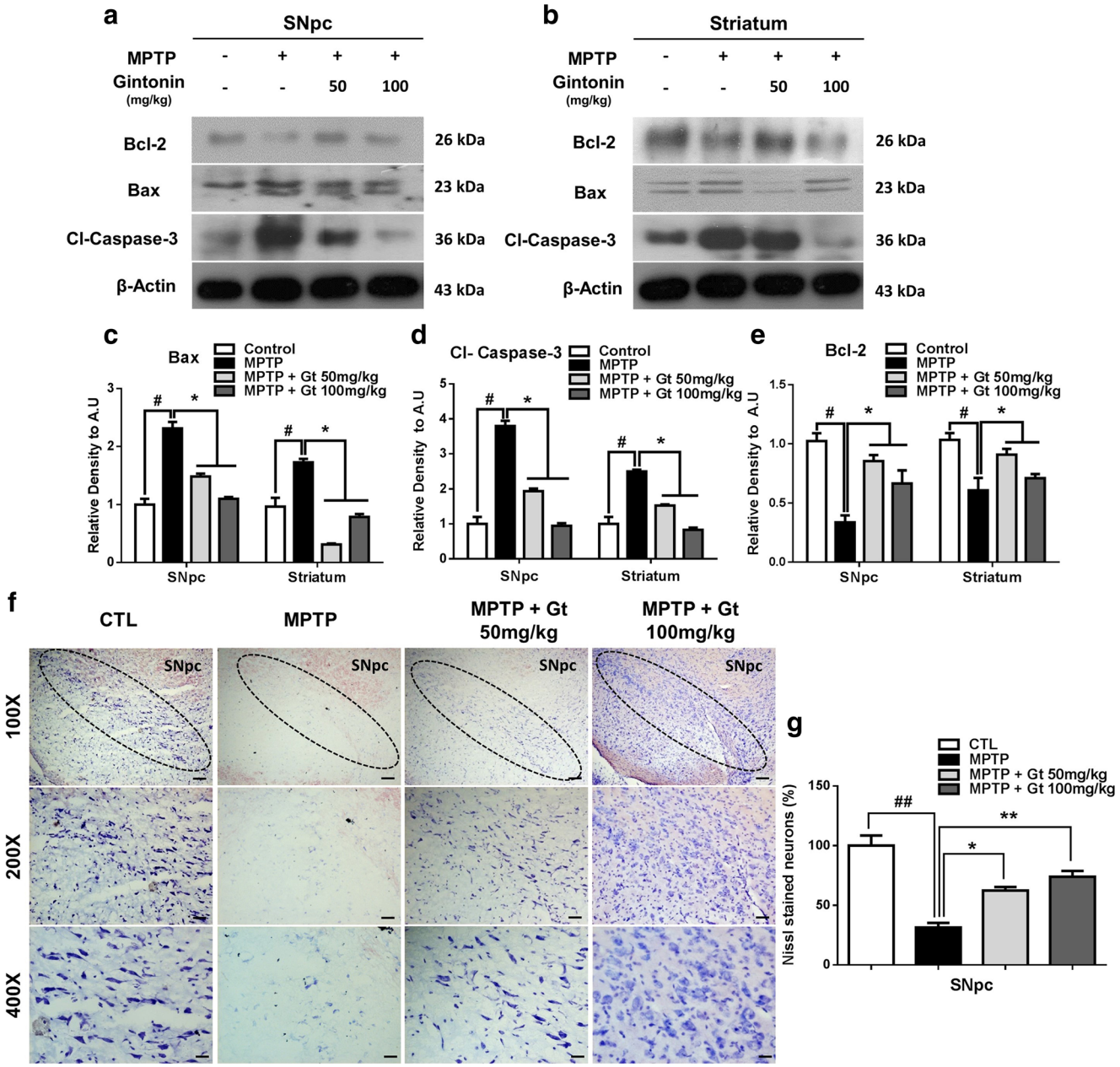


Fig. 5 Gintonin ameliorated the expression of apoptotic markers in the substantia nigra and striatum of MPTP-treated mice. **a**, **b** Images of the scanned immunoblot results of cleaved caspase-3, Bax, Bcl-2, and β-actin as a loading control in the substantia and striatum of the mouse brain. **c**–**e** Quantitative analysis of the effects of MPTP and gintonin on the expression of the anti-apoptotic marker Bcl-2 and pro-apoptotic markers Bax and cleaved caspase-3. **f** Images of the Nissl staining of the substantia nigra of different experimental groups captured at different magnifications. **g** Graphical representation of the Nissl-stained neurons in different experimental groups. The values are expressed as the mean ± SD for indicated proteins, and the number of independent experiments was $n = 10$ mice/group. #Significantly different from Veh-treated mice; *significantly different from MPTP-treated mice. Significance: $^{*}P < 0.05$; $^{\#}P < 0.05$

shown in Fig. 3c, d. To confirm that gintonin rescued the MPTP-induced oxidative stress effects, we performed ROS and LPO assays. Our findings indicated that with MPTP treatment, there were significantly higher ROS levels than in the vehicle-treated control group, while oral administration of gintonin significantly reduced ROS production in the substantia nigra and striatum brain homogenates (Fig. 3e, f).

Gintonin Inhibited the Expression of Inflammatory Mediators in the Substantia Nigra and Striatum In Vivo

Evidence has shown that microglia-imitative inflammatory and cytotoxic factors, including TNF-α, COX-2, and NOS-2, are also associated with the loss of nigrostriatal

dopaminergic neurons in the MPTP model of PD [62]. Thus, we determined whether gintonin regulates the expression of TNF-α, COX-2, and NOS-2 in the MPTP-treated mouse substantia nigra and striatum (Fig. 4a, b). Our western blot results showed that gintonin regulated MPTP-induced expression of TNF-α, COX-2, and NOS-2 in the substantia nigra and striatum (Fig. 4c–e).

Gintonin Regulated the Expression of Apoptotic Markers in the Substantia Nigra and Striatum of Mice

Apoptotic cell death is the main component of DA neurodegeneration [63]. Many studies have been conducted in this regard and have confirmed that MPTP causes apoptotic cell death [64]. Pro-apoptotic (Bax and caspase-3) and anti-

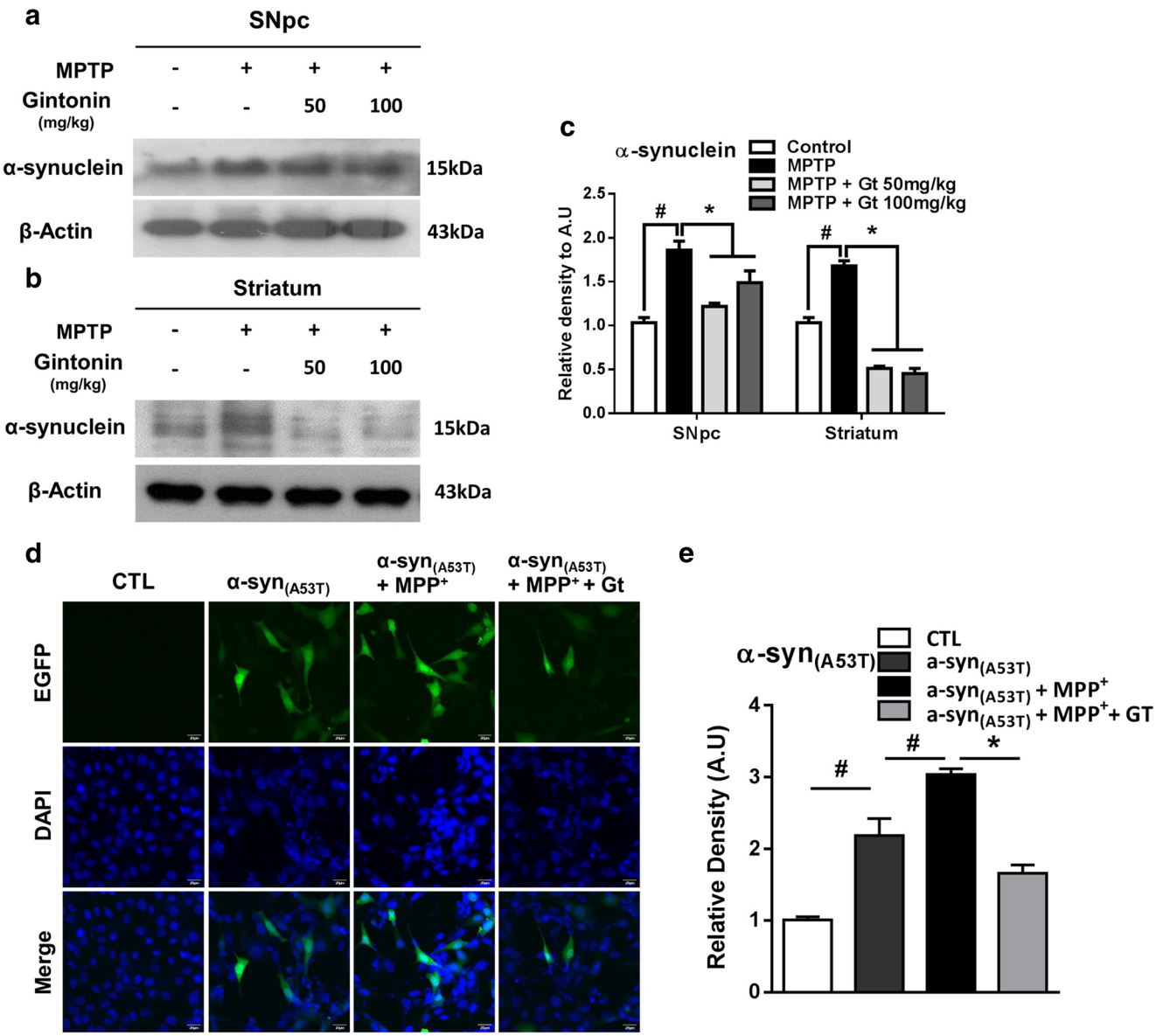


Fig. 6 Gintonin regulated the MPTP-induced expression of α-synuclein in the striatum and substantia nigra of the MPTP-treated mouse brain. **a**, **b** Images of the scanned immunoblot results of α-synuclein and β-actin as a loading control in the substantia and striatum of the brains of mice in different experimental groups. **c** Quantitative analysis of the effects of MPTP/gintonin on the expression of α-synuclein, which showed that the level of α-synuclein was significantly increased by MPTP treatment compared to that in vehicle-treated control mice. **d** Confocal microscopy images of SH-SY5Y cells in the control group, SH-SY5Y cells transfected with α-synuclein, and α-synuclein-transfected cells treated with MPP^+ and gintonin. **e** Graphical representation, showing the relative expression of α-synuclein in MPP^+-treated SH-SY5Y cells and the effects of gintonin on α-synuclein expression. #Significantly different from the control; #significantly different from α-synuclein transfected SH-SY5Y cells; *significantly different from MPP^+-treated cells and gintonin-treated group. Significance: $^*P < 0.05$; $\#P < 0.05$. The values are expressed as the mean ± SD for indicated proteins, and the number of independent experiments was $n = 10$ mice/group. #Significantly different from control mice; *significantly different from MPTP-treated mice. Significance: $^*P < 0.05$; $\#P < 0.05$

apoptotic (Bcl-2) markers as members of the Bcl-2 family are the primary controllers of the apoptotic cell death pathways in mitochondria [47]. Therefore, we studied the effects of gintonin on MPTP-induced apoptotic markers. According to our findings, gintonin at the doses of 50 and 100 mg/kg reversed the effects of MPTP, significantly attenuating the expression of pro-apoptotic markers caspase-3 and Bax and enhancing the expression of the anti-apoptotic marker Bcl-2 in the substantia nigra and striatum of the mouse brain, as shown in Fig. 5c–e. The effects of gintonin on neuronal degeneration were further evaluated by Nissl staining, which confirmed that gintonin increased the number of Nissl-stained substantia nigra neurons in MPTP-treated mice (Fig. 5f, g).

Gintonin Reversed MPTP-Induced α-Synuclein Expression in the Substantia Nigra and Striatum of the Mouse Model

Alpha synuclein has been identified as a prominent component of the Lewy bodies (LB), the pathological hallmark of PD [65]. Another study also confirmed that α-synuclein aggregation occurs in mouse and primate models of MPTP-induced Parkinsonism [66]. Because α-synuclein is a key player in the pathogenesis of Parkinson's disease, we analyzed the effects of gintonin (50 and 100 mg/kg, p.o. for 21 days) on the MPTP-induced aggregation of α-synuclein in the mouse brain substantia nigra and striatum. Per our findings, MPTP

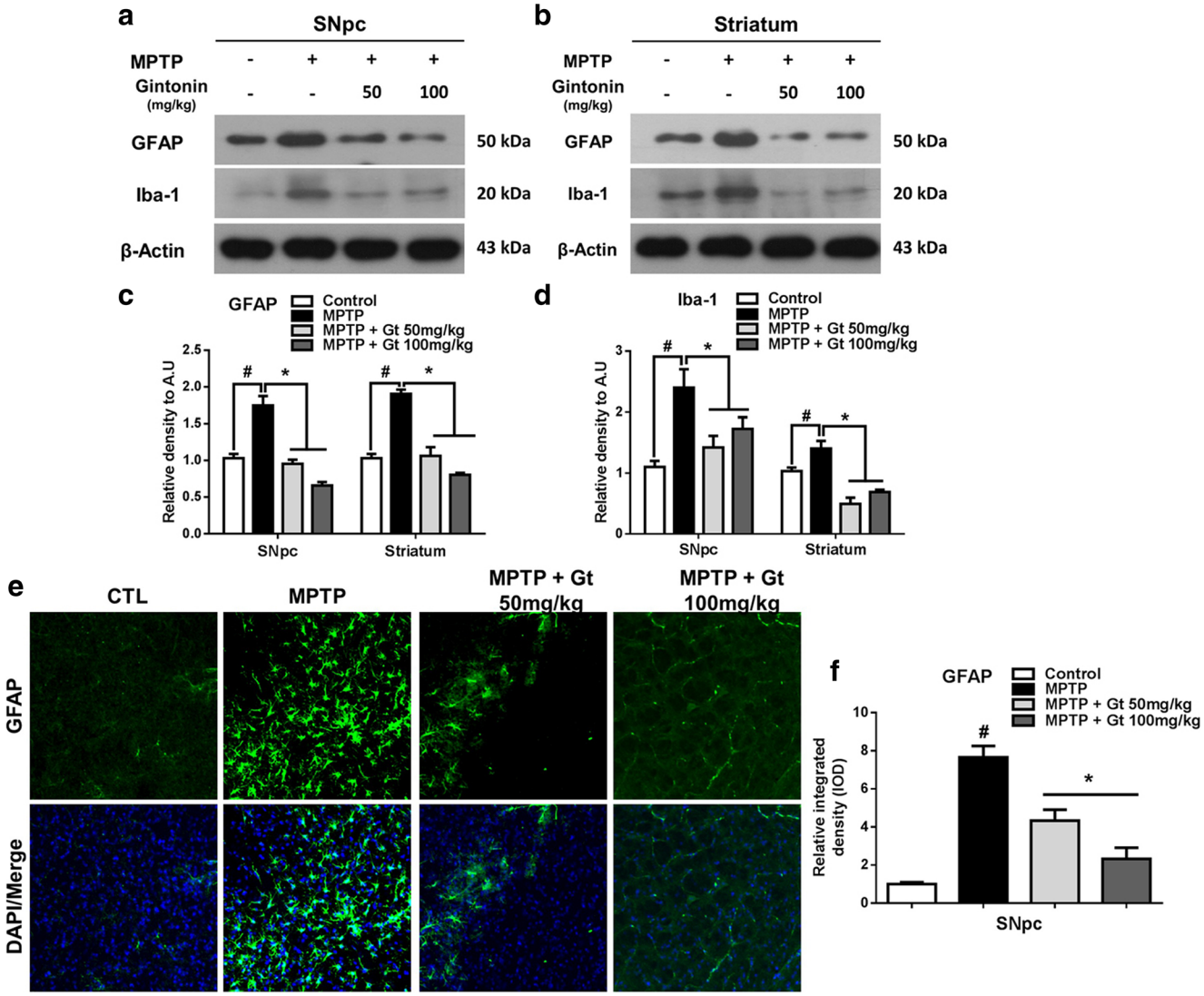


Fig. 7 Gintonin abrogated microgliosis and astrocytosis by regulating the expression of GFAP and Iba-1 in MPTP-treated mice. **a**, **b** Images of the scanned immunoblot results for GFAP and Iba-1 in the substantia nigra and striatum of different experimental mouse groups. **c**, **d** Histograms showing the differences among the different experimental groups ($n = 4$). **e**, **f** Representative images and the corresponding graph showing the immunofluorescence analysis of GFAP in the substantia nigra of the experimental groups. ImageJ software was used for immunohistological analysis. Values show the mean ± SD. *Significantly different from the control; #significantly different from MPTP-treated mice ($n = 10$ mice/group). Significance: $^{*}P < 0.05$; $^{\#}P < 0.05$

induced the expression of α-synuclein, and 100 mg/kg gintonin significantly abrogated the level of α-synuclein in the substantia nigra and striatum of the mouse brain, as shown in Fig. 6a–c. The beneficial protective effects of gintonin against abnormal α-synuclein accumulation were further confirmed by confocal microscopy, as shown in Fig. 6d.

Gintonin Mitigates MPTP-Induced Astrocytosis and Microgliosis

Previous studies have shown that there is elevated level of microgliosis and astrocytosis in the MPTP-treated mouse model [67]. GFAP and Iba-1 are the markers of triggered astrocytes and microglia, respectively [68]. According to our western blot results, there was an elevated expression of GFAP and Iba-1 in the MPTP-treated mice compared to the control mice. Administration of gintonin (50 and 100 mg/kg, p.o. for 21 days) to MPTP-treated mice significantly reduced the expression of GFAP and Iba-1 compared to MPTP treatment alone (Fig. 7c, d), showing a significant inactivation of abnormal microglia and astrocytes. Consequently, the confocal microscopic analyses indicated that GFAP immunofluorescence reactivity was significantly elevated in the substantia nigra of the MPTP-treated group compared to the control saline-treated group. Gintonin significantly abrogated the immunofluorescence in the substantia nigra of the MPTP-treated mice compared to MPTP treatment alone (Fig. 7e, f).

Protective Role of Gintonin Against MPP^+ /Rotenone-Induced Neurotoxicity In Vitro

Previous studies have shown that MPP^+ and rotenone cause neurotoxicity in human neuroblastoma SH-SY5Y cells and have been used in various PD-related studies [69]. Based on this hypothesis, we used MPP^+ at a dose of 2.5 mM and rotenone at doses of 500 and 200 nM. According to the in vitro

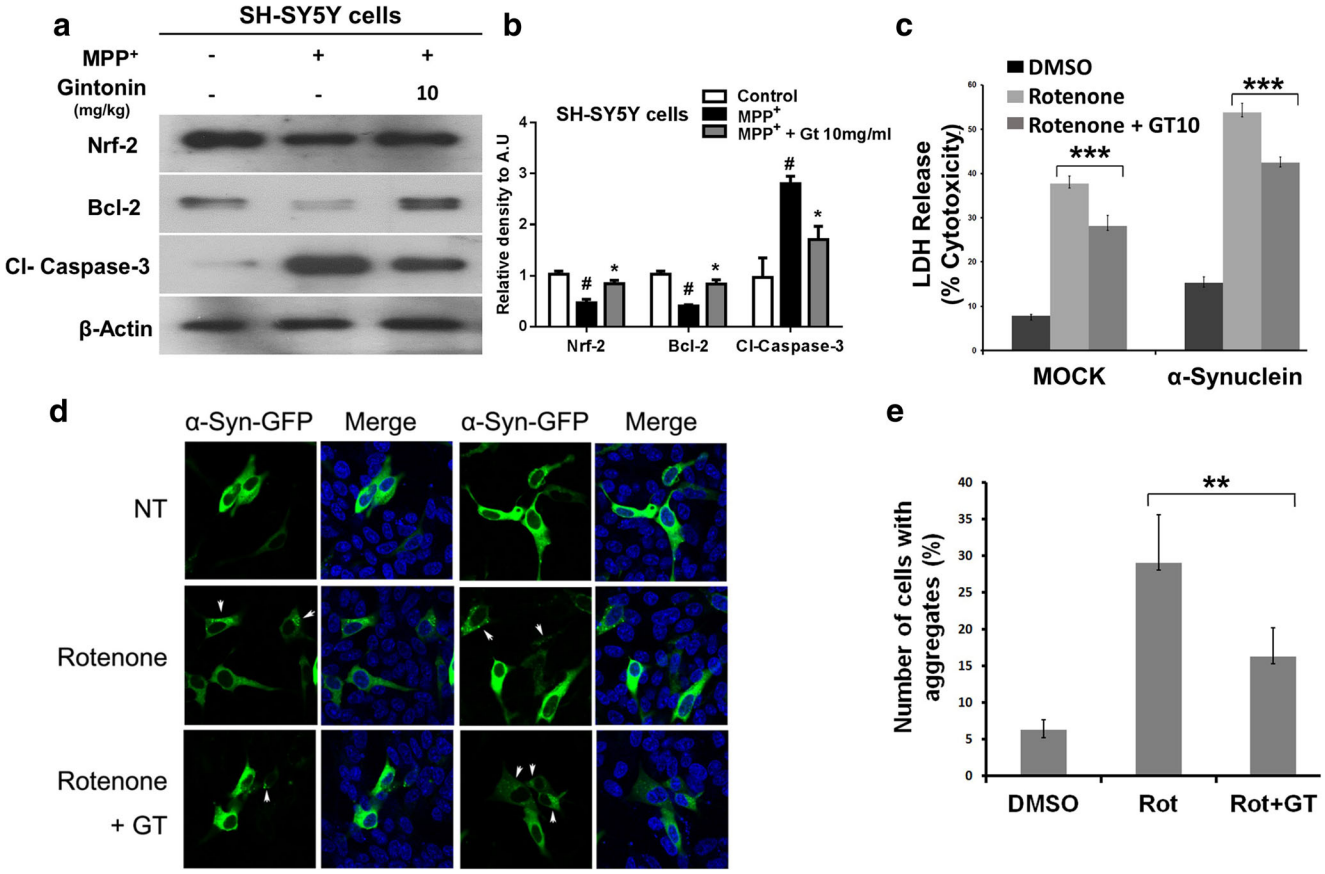


Fig. 8 Gintonin rescued the SH-SY5Y cells against MPP^+/rotenone-induced neurotoxicity in vitro. **a**, **b** Immunoblot results showing the expression of Nrf2, Bcl-2, and caspase-3 in SH-SY5Y cells following MPP^+ and gintonin treatment. The differences were quantified by using ImageJ software and shown by histograms. β-Actin was used as a loading control. **c** Graphical representation of the LDH assay. **d** Immunofluorescence analysis of the SH-SY5Y cells transfected with α-synuclein and treated with rotenone and gintonin. **e** Quantitative analysis of the effects of gintonin on rotenone-induced cytotoxicity. ImageJ software was used for immunohistological analysis. Values showing the mean ± SD. *Significantly different from the vehicle-treated control group; #significantly different from MPP^+-treated cells; #significantly different from the control group; *significantly different from the MPP^+-treated cells. Significance: $^*P < 0.05$; $\#P < 0.05$

studies, MPP^+ reduced the expression of Nrf2 and Bcl-2 and elevated the expression of caspase-3, which were significantly regulated with the administration of gintonin (10 μg/ml), as shown in Fig. 8a, b. Similarly, the LDH assay demonstrated that there was a significant increase in LDH release, an indicator of cell cytotoxicity, which was abrogated with the administration of gintonin, as shown in Fig. 8c. The beneficial effects of gintonin were further confirmed by confocal histological analysis; when rotenone was used to induce cytotoxicity, we found that gintonin significantly regulated the expression of α-synuclein in rotenone-treated α-synuclein-transfected SH-SY5Y cells, as shown in Figs. 8d, e and 9.

Discussion

In the current study, we for the first time provide the direct evidences that gintonin protected against MPTP-induced neurotoxicity in male C57BL/6N wild type mice. Previously, gintonin has been demonstrated to rescue depressive-like behaviors, improve the performance of mice in a rotarod test, and improve hippocampal-dependent cognitive impairments [38, 43]. Keeping in minds the protective roles of gintonin against different neurological disorders, we evaluated the effects of gintonin against MPTP-induced mice model of PD. To show the beneficial effects of gintonin, we evaluated the effects of gintonin against behavioral alterations, oxidative stress, apoptotic cell death, neuroinflammation, astrocytosis, and microgliosis.

Oxidative stress plays a critical role in the pathogenesis of PD. Candidate compounds that target mitochondrial oxidative stress are considered promising therapeutic agents for this disease. However, the use of exogenous antioxidants raises different problems concerning the impermeability of the blood-brain barrier, as well as secondary effects due to high doses needed to provide efficacy in long-term use [70]. In this regard, the need for alternative therapeutic strategies to counteract ROS-mediated damage and restore the redox cellular balance is of

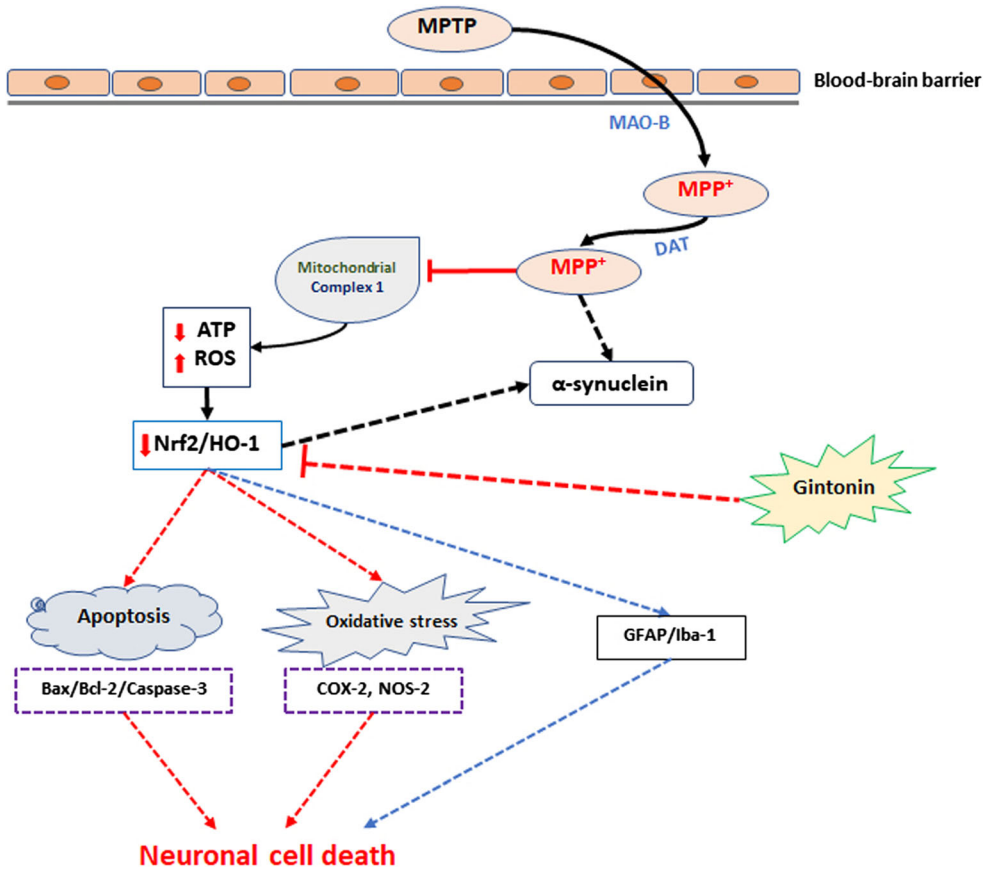


Fig. 9 Scheme of the possible mechanisms of the neuroprotective effects of gintonin in the MPTP mouse model of PD. A schematic diagram showing the involvement of the Nrf2/HO-1/GFAP/Bax pathway by which gintonin protects DA neurons from MPTP/MPP^+-induced neurotoxicity in in vivo and in vitro PD models. Once MPP^+ is taken into DA neurons through the dopamine transporter (DAT), this molecule induces ROS production due to mitochondrial complex I inhibition, which subsequently inhibits the Nrf2/HO-1 pathway. Ultimately, ROS overproduction and inhibition of Nrf2/HO-1 transactivates COX-2/NOS-2 expression, activates caspase-3/Bax, and induces cell death. However, treatment with gintonin upregulates the Nrf2/HO-1 pathway. Thus, by regulating the expression of Nrf2/HO-1, gintonin protects mitochondria from damage and eventually attenuates the MPTP/MPP^+-induced neurotoxicity in in vivo and in vitro PD models

utmost importance. A promising target against oxidative stress is the transcription factor Nrf2, which is responsible for inducing the expression of several endogenous cytoprotective enzymes. Consequently, the use of potential Nrf2 inducers may be a possible therapeutic strategy in PD [71].

Here, we first targeted the effects of gintonin against endogenous ROS regulators (Nrf2/HO-1 pathway), affected by administration of MPTP in the substantia nigra and striatum of C57BL/6 male mice. As the DA-expressing areas of the brain are more vulnerable to oxidative stress because of the reactive oxygen species (ROS) produced by DA metabolism [2]. Under conditions of oxidative stress, there is significant loss of Nrf2 and HO-1; to boost the endogenous antioxidant system, the regulation of Nrf2 and HO-1 is essential. Nrf2/HO-1 pathway activation results in the transcription of cytoprotective enzymes [70]. According to our findings, oral administration of gintonin significantly potentiated Nrf2 nuclear translocation and upregulated HO-1 expression levels in the MPTP treated mice, compared to the MPTP alone, suggesting the possible antioxidant effect of gintonin against MPTP-induced neurodegeneration. MPTP induces upregulation of the intracellular ROS levels in the mouse substantia nigra and striatum, as shown previously [71].

The antioxidant effects of gintonin were further evaluated by LPO and ROS assays, as MPTP induces upregulation of the intracellular ROS levels in the mouse substantia nigra and striatum, as shown previously [71]. The results of these assays showed that MPTP treatment was associated with an increase in ROS and lipid peroxidation levels in the substantia nigra and striatum of the mice, and these levels were significantly abrogated with gintonin treatment, showing the protective effects of gintonin against MPTP-induced neurodegeneration.

Another factor responsible for the degeneration of DA neurons is apoptosis. Previously, MPTP has been shown to activate apoptotic cell death machinery, which leads to dopaminergic neuronal cell death [72]. To assess the effects of gintonin on MPTP-induced neurodegeneration, we analyzed the expression of Bax, Bcl-2, and caspase-3 in the presence and absence of gintonin compared to the expression in control saline-treated mice. Our results showed that gintonin regulated the expression of apoptotic markers in the substantia nigra and striatum of the mice. The western blot results were further confirmed by confocal microscopy.

GFAP is a branded marker for reactive astrocytes in response to any sort of CNS injury, due to its specificity to astrocytes [73], while Iba-1 is a marker of activated microglial cells; both of the markers have been shown to be upregulated with MPTP treatment. We assessed the effects of gintonin on MPTP-induced activated gliosis and astrocytosis, and according to our findings, gintonin significantly reversed the expression of GFAP and Iba-1 in the substantia nigra and striatum of the mice, which was further confirmed by confocal microscope.

In conclusion, we elucidated for the first time that the Nrf2/HO-1 pathway is a biological therapeutic target of gintonin from our in vivo and in vitro findings. In addition, gintonin was shown to ameliorate the PD-like pathology, as assessed by biochemical, behavioral, and morphological analyses in in vivo and in vitro Parkinson's disease models. The pharmacological modulation of the Nrf2/HO-1 pathway by gintonin culminates in the upregulation of different cytoprotective enzymes, highlighting the possibility of the beneficial effects of gintonin in human PD and supporting its clinical application.

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Author Contributions MGJ: Concept design, data collection and assembly, data analysis and interpretation, animal grouping and treatment, and manuscript preparation. MI: Concept, design, data collection, analysis and interpretation, data assembly, and manuscript preparation. MHJ: conception, data collection, and analysis and interpretation. LY: in vitro data collection and analysis. KCC: in vitro data collection and analysis. SN: provision of gintonin and critical idea for in vivo experiments. HH: basic in vitro data analysis. HR: provision and managing of critical idea for experiments and basic in vitro data analysis. MOK: provision of study material, data analysis and interpretation, administrative support, critical reading of manuscript and final approval of manuscript.

Compliance with Ethical Standards

The animals used in the experiment were handled in accordance with the animal ethics committee (IACUC) guidelines issued by the Division of Applied Life Sciences, Department of Biology at Gyeongsang National University, South Korea. The methods used for animal's experiments were in accordance with the already-approved guidelines (Approval ID 125) by the animal ethics committee (IACUC) of the Division of Applied Life Sciences, Department of Biology at Gyeongsang National University, South Korea.

Conflict of Interest The authors declare that they have no competing interests.

Abbreviations DA, Dopamine; DMSO, Dimethyl sulfoxide; FBS, Fetal bovine serum; HO-1, Heme oxygenase-1; NOS-2, Nitric oxide synthase-2; MPP^+, 1-Methyl-4-phenylpyridinium; MPTP, 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine; Nrf2, Nuclear factor erythroid 2-related factor 2; PD, Parkinson's disease; ROS, Reactive oxygen species; SNpc, Substantia nigra pars compacta; STR, Striatum; TH, Tyrosine hydroxylase; TNF-α, Tumor necrosis factor-α; COX-2, Cyclo-oxygenase-2; LPO, Lipid peroxidation; Gt, Gintonin

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