Protective effects of harpagoside on mitochondrial functions in rotenone-induced cell models of Parkinson's disease

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Abstract. Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease. Currently, no radical treatment is available for this disease. Harpagoside is a proposed neuroprotective iridoid active ingredient that can be derived from *Scrophulariae buergeriana, Scrophularia striata and Harpagophytum procumbens*. The present study aimed to investigate the effects of harpagoside on mitochondrial functions in rotenone-induced cell models of Parkinson's disease (PD). Neuro-2A (N2A) cells were treated with rotenone to establish *in vitro* cell models of PD. Cell viability and survival were measured using a Cell Counting Kit-8 assay. Biochemical assays with spectrophotometry were used to measure complex I activity, mitochondrial swelling and caspase 3 activity. The cell survival rate was first found to be significantly decreased by rotenone (20 nmol/l) treatment. However, intervention with harpagoside (10 μmol/l) was found to increase the cell survival rate of rotenone-induced N2A cell models differentiated with 1 mmol/l of dibutyryl-cAMP. At ≥0.1 μmol/l concentration, harpagoside significantly alleviated rotenone-induced mitochondrial swelling, whereas at 1 μmol/l it significantly counteracted the inhibitory effects of rotenone on complex I activity. At 10 μmol/l harpagoside significantly inhibited rotenone-induced caspase 3 activation. These results suggest that harpagoside has the potential to protect mitochondrial functions against rotenone-induced injury in N2A cell models of PD.

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Introduction

Parkinson's disease (PD) is a neurodegenerative disorder that is common in the elderly, ranking second only to Alzheimer's disease worldwide (1), with an incidence of ~1% in individuals aged >60 years and ~3% in individuals aged >80 years in developed countries (2,3). The pathological features of primary PD include a reduction in dopamine (DA) neurons in the dense region of the substantia nigra and degeneration and spherical eosinophilic granule Lewy bodies in the cytoplasm (4,5). A number of studies have previously found that mitochondria serve an important role in the pathogenesis of PD (6,7). Levodopa is a dopamine precursor, the tablet of which is widely regarded to be the gold standard treatment for PD worldwide (8). The current treatment paradigms for PD remain focused on symptomatic management and has not reached the ideal clinical therapeutic effects (9,10). However, various neuroprotective agents, such as hyperoside (11), walnut oil (12), tribuli fructus (13) and nutraceuticals (Betanin) (14), are currently at the preclinical research phase (11-14). Although there is currently no cure for PD, pharmacological interventions, surgical treatments (deep brain stimulation, spinal cord stimulation for gait symptoms) (15) and other therapeutic approaches, such as magnetic resonance-guided focused ultrasound (15) and stem cell therapies (16), can alleviate motor (such as resting tremor, myotonia and bradykinesia) and non-motor (such as sensory impairments, autonomic nervous system dysfunction and psychoemotional disorders) symptoms effectively (17).

Although the etiology of PD has not been fully elucidated, genetic and environmental factors are considered to serve key roles in its development. Among the environmental factors, pesticide exposure is an important candidate for PD pathogenesis, including rotenone (18). Previous studies found that a rat model treated with rotenone injected intramuscularly could replicate two signature pathological features of PD, including the degeneration of substantia nigra DA neurons, formation of Lewy bodies in surviving substantia nigra neurons and PD-like dyskinesia (19,20). In addition, paraquat, 6-hydroxydopamine, 1-methyl-4-phenylpyridinium (MPP^+) have been utilized to treat Neuro-2A (N2A) cells for the construction of *in vitro* PD models (21,22). Subsequent studies have demonstrated that rodent models treated with rotenone exhibit

extranigral pathological and non-motor symptom changes, such as hyposmia, gastrointestinal dysfunction, sleep disturbances, circadian dysfunction, cognitive decline, depression and anxiety (19,23,24).

Harpagoside is an iridoid active ingredient that can be derived from *Scrophulariae buergeriana, Scrophularia striata* and *Harpagophytum procumbens* (HP) (25-27). It was previously found to have neuroprotective effects. Numerous studies have previously demonstrated the neuroprotective effects of plant extracts containing harpagoside against damage in both cultured cell models and animal models. *Radix Scrophulariae,* dried roots of *Scrophularia ningpoensis Hemsl,* was reported to exert neuroprotection against cerebral ischemia and reperfusion injury through the ERK1/2 and p38 MAPK pathways (25). Furthermore, *Scrophularia buergeriana* extracts (SBE) demonstrated neuroprotective properties against glutamate-induced cytotoxicity in SH-SY5Y cell models through its antioxidant and anti-apoptotic mechanisms (26). SBE also attenuated neuroinflammation in BV-2 microglial cells induced by lipopolysaccharide and promoted neuroprotection in SH-SY5Y neuroblastoma cells treated with lipopolysaccharide-induced BV-2 conditioned media according to another previous study (27). The application of SBE to SH-SY5Y cells significantly improved their viability in the presence of glutamate by promoting the expression of various antioxidant proteins, such as superoxide dismutase (SOD)1, SOD2 and glutathione peroxidase-1, in addition to directly augmenting total glutathione levels (26). In addition, SBE was observed to reduce DNA damage and diminish the activation of Bcl-2-associated X protein, cleaved caspase-3 and cleaved poly[adenosine diphosphate (ADP)-ribose] polymerase (26). SBE also increased Bcl-2 expression in a glutamate-induced SH-SY5Y cell model through p38 MAPKs (26). *Scrophularia striata* exhibited antioxidant and neuroprotective properties against neurotoxicity in PC12 cells treated with H_2O_2 (28). The high-polarity methanolic fraction of the aerial parts of *Scrophularia striata* demonstrated significant neuroprotective activity against glutamate-induced neurotoxicity in rat cerebellar granule neurons in a dose-dependent manner in a previous *in vitro* study (29). Aerial parts of the plant were air-dried, powdered and macerated with an 80% ethanol solution for 3 days with three changes of the solution (29). In addition, harpagide derived from *Scrophularia* protected rat cortical neurons against injury induced by oxygen-glucose deprivation and subsequent reoxygenation through the reduction of endoplasmic reticulum stress (30). Harpagoside was also effective in restoring both spatial learning and memory in addition to fear memory impairments in rats with chronic cerebral hypoperfusion (31). Additionally, harpagoside enhanced the activity of Akt whilst inhibiting the activity of GSK-3β in the hippocampal homogenates of rats with chronic cerebral hypoperfusion, which are downstream effectors of PTEN (31). Sun *et al* (32) previously found that harpagoside alleviated 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)/MPP^+-induced DA neurodegenerative changes and movement disorders by enhancing the expression of glial cell line-derived neurotrophic factor (GDNF) in both cultured mesencephalic neurons induced by MPP^+ and a chronic MPTP-treated mouse model.

Blocking the GDNF signaling pathway partially, rather than completely blocking, all of the anti-PD effects of harpagoside on TH-positive neurons in the cultured mesencephalic neurons induced by MPP^+ (32,33). Therefore, there may be other pathways involved. Numerous studies have demonstrated that mitochondria serve an important role in PD pathogenesis (34,35). Li *et al* (36) previously found that harpagoside regulated mitochondrial function with concomitant of diminished mitochondrial oxidative damage and recovered mitophagy flux through p53/Parkin-mediated mitophagy in a model of doxorubicin-induced cardiotoxicity. Consequently, the present study hypothesized that the mitochondrial pathway may be one of the routes in which harpagoside can exert its effects on neurons. The present study investigated the effects of harpagoside on rotenone-induced mitochondrial damage in N2A cells.

Materials and methods

Cells. N2A cells (Cat. no. TCM29) were obtained from the The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences.

Main reagents. Harpagoside (Fig. 1) was obtained from Chengdu Pusen Biotechnology Co. Ltd, China. Rotenone and db-cAMP were obtained from the Sigma-Aldrich; Merk KGaA. Minimum Essential Medium (MEM), FBS and trypsin were acquired from Invitrogen; Thermo Fisher Scientific, Inc. The mitochondrial isolation kit (cat. no. C3601) and caspase 3 detection kits (cat. no. C1115) were obtained from Beyotime Institute of Biotechnology, China. Cell Counting Kit-8 (CCK-8) was procured from the Dojindo Laboratories, Inc. NADH, potassium ferricyanide, Triton X-100 and HEPES were obtained from Shanghai Aladdin Biochemical Technology Co., Ltd, China.

Methods

N2A cell culture and differentiation. N2A cells, a neuroblastoma cell line originally derived from the spontaneous brain tumor of the mouse neural crest (37), were cultured in MEM containing 50 U/ml penicillin, 50 μg/ml streptomycin and 10% FBS. Depending on the experimental requirements, cells were seeded at a specific density in 96- or 6-well cell culture plates and incubated at 37˚C in a 5% CO_2 cell culture incubator with saturated humidity. Since N2A cells exhibit distinct tumorigenic characteristics and lack certain features of dopaminergic neurons (37,38), it is necessary to induce their differentiation (37,39) for PD modelling. According to previous studies (37,40), N2A cell differentiation was induced by reducing FBS concentrations in the presence/absence of retinoic acid or dibutyryl-cAMP (db-cAMP). FBS concentration in the medium was reduced from 10 to 0.5%, whilst 1 mmol/l of db-cAMP was added to culture system overnight at 37˚C to obtain differentiated N2A cells, which were confirmed by the enhanced expression of tyrosine hydroxylase (TH) as demonstrated by immunocytochemistry (37) and utilized for subsequent experimental investigations.

Establishment of cell models induced by rotenone. N2A cells were seeded on a 96-well plate at a density of

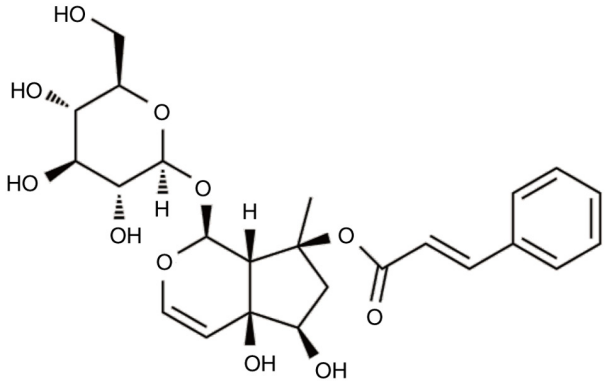

Figure 1. Chemical structural formula of harpagoside.

$5x10^4$ cells/ml. Each well contained 200 μl cell suspension. After overnight incubation at 37˚C to allow cell attachment, cells were treated at 37˚C with 20 nmol/l rotenone for 48 h to establish cell models.

Cell viability assay. Cell viability was determined using an assay kit, according to the manufacturer's instructions. After the N2A cells were incubated for 48 h at 37˚C, 10 μl CCK-8 reagent was added to each well and cultured in a 5% CO_2 incubator at 37˚C for 2 h. A measurement wavelength of 450 nm and a reference wavelength of 620 nm were measured using a microplate reader. The cell survival rate was calculated using the following formula: Cell survival=(As-Ab)/(Ac-Ab). In this formula, As represents the test cell medium, CCK-8 and cytotoxic substances, Ac represents the control cell culture medium, CCK-8 and no cytotoxic substances. By contrast, Ab represents the blank well, with no cells or toxic substances but with cell culture medium and CCK-8. Three independent replicate experiments were performed.

Effects of harpagoside on rotenone-induced N2A cells. The experiment was divided into the following five groups: Control, Model, model with low, medium and high doses of harpagoside. N2A cells were seeded in a 96-well plate at a density of $5x10^4$ cells/ml. Each well contained 200 μl cell suspension. After 12 h incubation at 37˚C, different harpagoside concentrations (0.1, 1 and 10 μmol/l) were added and incubated at 37˚C for 2 h. Rotenone (20 nmol/l) was then added and incubated at 37˚C for 48 h. The model group received only rotenone for injury induction, whereas the control group did not receive rotenone or harpagoside treatment. Cell viability was then assessed using the CCK-8 reagent.

Effects of harpagoside on rotenone-induced mitochondrial complex I activity in N2A cells. For cell culture, N2A cells were seeded at a density of $5x10^4$ cells/ml. After 48 h incubation at 37˚C, $2x10^7$ cells were collected for mitochondrial preparation. For mitochondrial preparation, cells were gently suspended in ice-cold PBS, centrifuged at 600 x g at 4˚C for 5 min and the supernatant was removed. For pre-treatment, phenylmethyl sulfonyl fluoride was mixed with the mitochondrial isolation reagent before being added to the cell pellet. The cells were suspended and incubated on ice for 15 min, before homogenization, where the cell suspension was transferred to a glass homogenizer and homogenized ~20 times. The homogenization efficiency was then determined. After homogenization for 10 times, 2 μl homogenized solution was added to 30 μl Trypan Blue staining solution and mixed, before the proportion of blue-stained cells (positively stained) was observed under a light microscope. If <50% of the cells were positively stained, homogenization would be increased five times and observed again. If ≥50% cells were positively stained, homogenization would be stopped, since excessive homogenization may cause mechanical damage to mitochondria. The number of homogenizations used for the subsequent experiments was then recorded. The cell lysate was then centrifuged at 600 x g and 4˚C for 10 min. The supernatant was transferred to another centrifuge tube and centrifuged again at 11,000 x g and 4˚C for 10 min. The supernatant was discarded and the pellet contained isolated mitochondria.

For the measurement of mitochondrial complex I activity, the experiment was divided into four groups: Control, Model, model with low and high doses of harpagoside. The final harpagoside concentrations were 0.1 and 1 μmol/l in the model group treated with low- and high-dose harpagoside, respectively. Buffer for mitochondrial complex I activity measurement contained NADH (0.17 mmol/l), potassium ferricyanide (0.6 mmol/l) and Triton X-100 (1 ml/l), dissolved in PBS (pH 7.4). The mitochondrial complex I activity measurement reaction system contained 35 μl detection buffer, 5 μl mitochondrial lysate, 5 μl ubiquinone solution and 5 μl rotenone. The addition of mitochondria served as the starting time for the reaction, followed by incubation at 30˚C in a water bath for 10 min. The absorbance of NADH was measured at 340 nm using a spectrophotometer. A standard curve was simultaneously obtained. The unit definition for mitochondrial complex I activity is the number of nmol NADH oxidized per min per mg mitochondrial proteins. The levels of mitochondrial proteins were determined by BCA protein assay kit.

Effect of harpagoside on rotenone-induced mitochondrial swelling in N2A cells. For cell culture, the experiment was divided into the following six groups: Control, Model and model containing 0.01, 0.1, 1 and 10 μmol/l harpagoside. N2A cells were seeded at a density of $1x10^6$ cells/ml in a six-well plate at 2 ml/well. After 12 h of incubation at 37˚C, harpagoside was added at final concentrations of 0.01, 0.1, 1 and 10 μmol/l. After 2 h of incubation at 37˚C, rotenone (20 nmol/l) was added and the cells were cultured for 9 h at 37˚C.

To detect mitochondrial swelling, cells were collected to prepare mitochondrial samples using a buffer solution for swelling detection. Solutions A and B were then used. Solution A contained 125 mmol/l sucrose, 65 mmol/l KCl, 10 mmol/l HEPES/KOH (pH 7.4) and 5 mmol/l potassium succinate. Solution B contained 10 mmol/l $CaCl_2$. The reaction system for this detection consisted of the ratios of Solution A: Solution B: samples=50:10:40 μl, which was utilized at room temperature. Absorbance was then measured at 540 nm, starting with adding $CaCl_2$ and readings were obtained every 2 min for 10 min. Mitochondrial swelling was estimated as a decrease in absorbance within 10 min.

Effect of harpagoside on rotenone-induced caspase 3 activity in N2A cells. The experiment was divided into the following four groups: Control, Model, Model treated with low and high doses of harpagoside. Harpagoside concentrations were 1 and 10 μM in the low-dose and high-dose groups, respectively. A density of $2x10^5$ cells/ml N2A cells was seeded

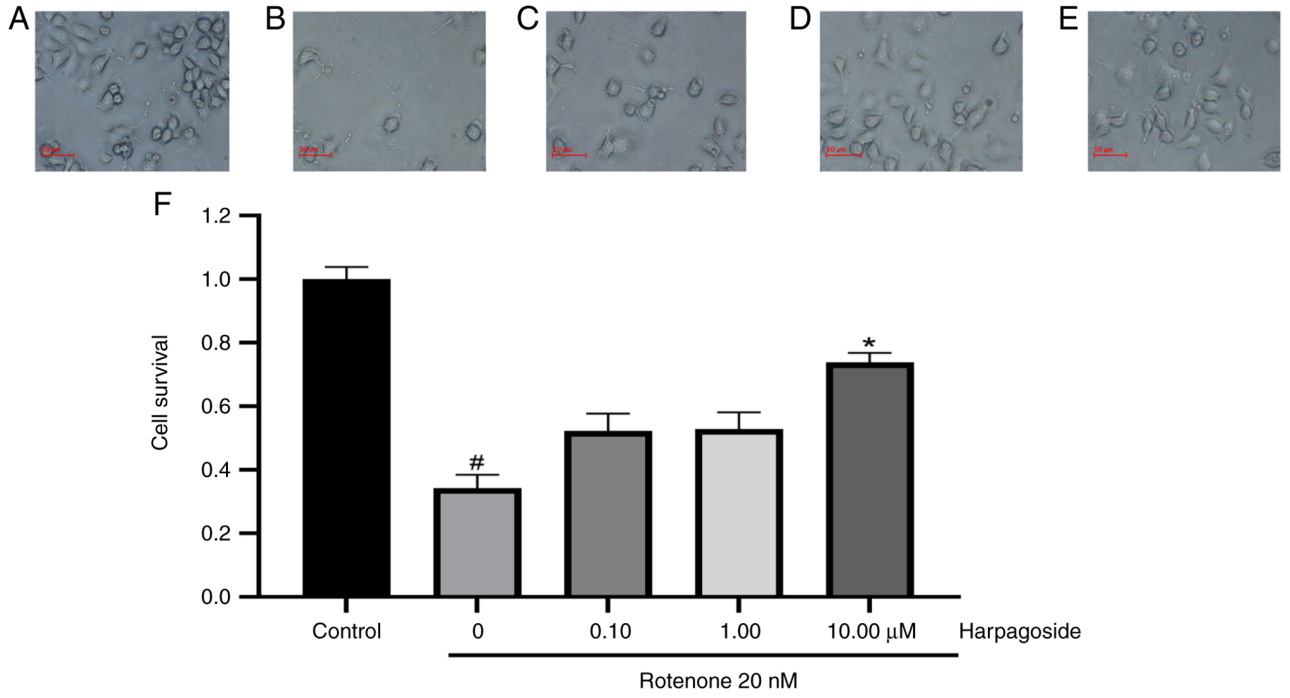

Figure 2. Protective effects of harpagoside on rotenone-induced effects in N2A cells. Data are expressed as mean ± standard error of the mean (n=4). Representative image of N2A cell cultures under a light microscopy. (A) Control group, (B) Rotenone-treated group, Group treated with rotenone and (C) 0.1, (D) 1 or (E) 10 μmol/l harpagoside respectively. (F) Cell Survival Rate. $^{\#}$P<0.05 vs. Control; *P<0.05 vs. Model group.

into six-well plates. After 12 h of incubation at 37˚C, harpagoside was administered at predetermined concentrations. After 2 h of incubation at 37˚C, rotenone (20 nmol/l) was added and the cells were cultured for 24 h at 37˚C.

For sample processing, N2A cells were collected using a cell scraper and centrifuged at 600 x g and 4˚C for 5 min. The cells were washed with PBS and centrifuged under identical conditions to obtain cell pellets. Subsequently, 100 μl cell lysis buffer (cat. no. C1115-1; contained within the kit) obtained from Beyotime Institute of Biotechnology, was added to cell pellets. The cell pellets were then resuspended, lysed in an ice bath for 15 min and centrifuged at 16,000 x g and 4˚C for 10 min. The supernatant was analyzed to measure the caspase 3 activity using the caspase-3 activity assay. The Caspase 3 activity detection kit is based on the ability of caspase 3 to catalyze the substrate acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA), resulting in yellow p-nitroaniline (pNA) production. Consequently, the enzymatic activity of caspase 3 was quantified by measuring the absorbance, since pNA exhibited strong absorption at ~405 nm. The reaction system setup included adding detection buffer (80 μl), sample to be tested (10 μl) and substrate Ac-DEVD-pNA (2 mmol/l; also added as 10 μl), resulting in a total volume of 100 μl. First, the detection buffer was added, before the sample was introduced to ensure proper mixing without bubbles before adding Ac-DEVD-pNA. The mixture was incubated at 37˚C for 120 min. The blank control was substituted with a detection buffer for the sample. Second, the absorbance was measured at 405 nm. The absorbance of pNA catalyzed by caspase 3 in the sample was calculated by subtracting the absorbance of the blank control from that of the sample. Third, caspase-3 enzyme activity was calculated as the quantity of enzyme required to catalyze 1 nmol Ac-DEVD-pNA to pNA per h at 37˚C, defined as one unit of enzyme activity.

Statistical analysis. Statistical analysis was performed using the SPSS (version 18.0; SPSS, Inc.). Data are presented as mean ± standard error of the mean. Multiple groups were compared using a one-way analysis of variance. If there was statistical significance, pairwise comparisons were conducted using the Tukey method. P<0.05 was considered to indicate a statistically significant difference.

Results

Protective effect of harpagoside on rotenone-induced N2A cells. Fig. 2 presents the effect of harpagoside on rotenone-induced N2A cells. The cell survival rate, one of the key indicators of cell viability, was 1.000±0.039 in the control group, which was decreased by 65.8% to 0.342±0.042 (P<0.05) after rotenone (20 nmol/l) treatment. Intervention with harpagoside (10 μmol/l) significantly increased the cell survival rate, to 0.738±0.030 (P<0.05), compared that in the model group. However, cell survival rates did not differ significantly between the model group and those treated with low and medium doses of harpagoside (0.1 or 1 μmol/l).

Effect of harpagoside on mitochondrial complex I activity in rotenone-induced N2A cells. Fig. 3 illustrates the effect of harpagoside on mitochondrial complex I activity in rotenone-treated N2A cells. Rotenone (2.5 μmol/l) significantly inhibited mitochondrial complex I enzyme activity, decreasing from 29.04±0.92 in the control group to 19.93±2.31 U/mg in the model group, resulting in a 31.37% decrease (P<0.05). Harpagoside (1 μmol/l) significantly reversed the

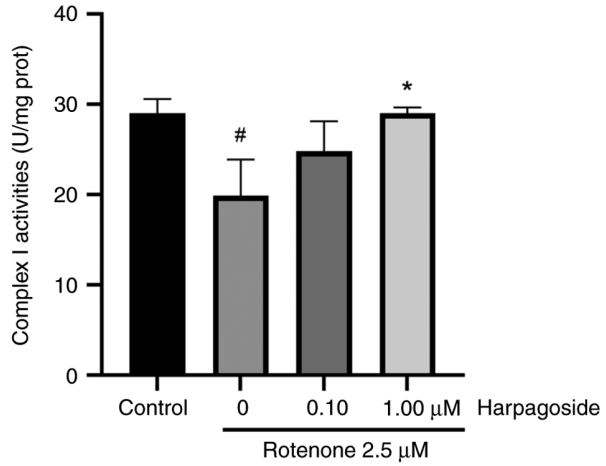

Figure 3. Effects of harpagoside on rotenone-induced mitochondrial complex I activity. Data are expressed as mean ± standard error of the mean (n=3). #P<0.05 vs. Control; *P<0.05 vs. Model group.

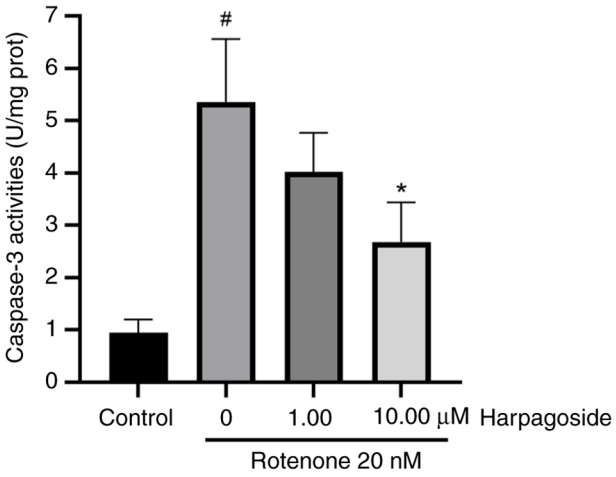

Figure 5. Effects of harpagoside on rotenone-induced caspase 3 activities. Data are expressed as mean ± standard error of the mean (n=3). #P<0.05 vs. Control; *P<0.05 vs. Model group. Prot, protein.

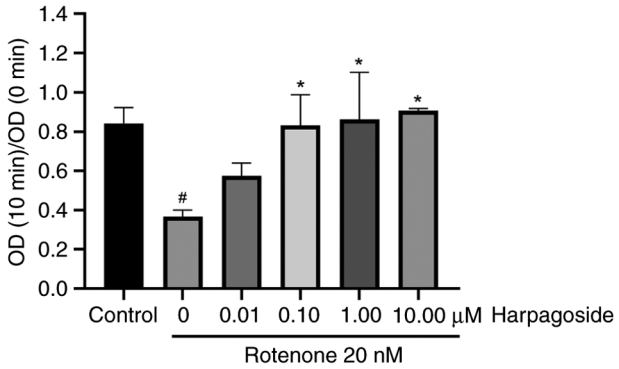

Figure 4. Effects of harpagoside on rotenone-induced mitochondrial swelling. Data are expressed as mean ± standard error of the mean (n=3). #P<0.05 vs. Control; *P<0.05 vs. Model group. OD, optical density; prot, protein.

rotenone-induced inhibitory effects on complex I, increasing its enzyme activity from 19.93±2.31 to 29.03±0.38 U/mg (P<0.05). However, treatment with lower doses of harpagoside (0.1 μmol/l) did not significantly affect the rotenone-induced inhibition of complex I.

Effect of harpagoside on rotenone-induced mitochondrial swelling in N2A cells. Fig. 4 presents the effects of harpagoside on rotenone-induced mitochondrial swelling in N2A cells. $CaCl_2$ solution was added to the mitochondrial swelling detection system for the mitochondrial swelling detection experiment. If the permeability of the mitochondria was strong, the absorbance at 540 nm would then be gradually decreased with a larger magnitude. The detection interval was 10 min and the degree of mitochondrial swelling was expressed as optical density (OD; 10 min)/OD (0 min) at the end of the measurement. A smaller ratio indicated a greater degree of swelling. In the control group, the OD (10 min)/OD (0 min) was 0.842±0.046, whilst treatment with rotenone (20 nmol/l) for 9 h resulted in a decreased ratio of 0.366±0.019 (P<0.05). Pre-treatment with harpagoside (0.1, 1 or 10 μmol/l) for 2 h significantly increased the rotenone-induced mitochondrial OD (10 min)/OD (0 min) from 0.366±0.019 to 0.831±0.090, 0.861±0.139 and 0.907±0.006, respectively (all P<0.05, respectively). Harpagoside (0.01 μmol/l) did not affect the degree of rotenone-induced mitochondrial swelling.

Effect of harpagoside on rotenone-induced caspase 3 activity in N2A cells. Fig. 5 presents the effect of harpagoside on rotenone-induced caspase 3 activity in N2A cells. The caspase 3 enzyme activity was 0.95±0.15 U/mg in the control group. However, treatment with rotenone for 24 h significantly increased caspase 3 activity to 5.36±0.69 U/mg (P<0.05). Pre-treatment with harpagoside (10 μmol/l) for 2 h significantly inhibited rotenone-induced caspase 3 activation (P<0.05), reducing its enzyme activity to 2.68±0.44 U/mg. After intervention with harpagoside (1 μmol/l), the caspase 3 enzyme activity was 4.02±0.43 U/mg, but no significant difference compared with that in the model group could be found.

Discussion

The pathogenesis of PD is complex. Oxidative stress, mitochondrial dysfunction and abnormal protein overexpression and aggregation have all been documented to be involved in the degeneration and deletion of substantia nigra DA neurons and the reduction of striatum DA levels (41,42). In particular, mitochondria-dependent apoptosis is a key factor in PD-related DA neurodegeneration. Age-related mitochondrial changes, including those in mitochondrial DNA, are strongly associated with PD (43). Mitochondrial energy disturbances and changes in mitochondrial distribution in neurons are also important factors in PD pathogenesis (44,45). Previous PD genomics studies have revealed that mitochondrial dysfunctions caused by bioenergetic defects, mutations in mitochondrial DNA, nuclear DNA gene mutations linked to mitochondria, are important aspects of the pathogenesis of PD (46,47). A number of familial PD-related pathogenic proteins can interact directly or indirectly with mitochondria (48,49). The proteins encoded by several PD-related genes, including α-synuclein, Parkin, PTEN-induced kinase 1, protein deglycase, leucine-rich repeat kinase 2 and serotonin receptor 2A, can be localized to the mitochondria. The aggregation of these proteins may cause

mitochondrial DNA damage and dysfunction (50). It has been previously proposed that the decreased activity of complex I in the mitochondrial respiratory chain in patients with PD and neurotoxin rotenone injury can cause abnormal energy production function, oxidative stress and mitochondria-dependent apoptosis in rat models of PD (46,51).

N2A cells have been extensively utilized to investigate neuronal differentiation, axonal growth and associated signaling pathways (37,52). A notable feature of this cell type is their capacity to differentiate into neurons within a matter of days (37,52). Although various treatments, such as serum deprivation, retinoids and bone morphogenetic proteins, can be used to generate N2A neurons, only db-cAMP can significantly promote the formation of DA neurons (37). Treatment of N2A cells with 1-4 mmol/l dbcAMP resulted in extensive differentiation and neurite outgrowth (53). Both TH and DA levels were previously demonstrated to significantly elevate in the presence of db-cAMP, as demonstrated by Western blotting (WB), immunocytochemistry and high-performance liquid chromatography (HPLC) (37). Specifically, WB analysis revealed that TH was endogenously expressed in N2A cells at detectable levels, where it was significantly (>2.5-fold) enhanced upon treatment with db-cAMP (37). The immunohistochemistry results indicated that strong TH expression was noted in individual N2A cells, which formed large colonies upon db-cAMP treatment for 3 days (37). The HPLC detection results revealed that N2A cells expressed significant levels of DA, which was further augmented upon db-cAMP treatment (37). N2A cells has been extensively used to study PD (54,55). N2A exhibited greater sensitivity to the lethal effects of MPP^+ compared with human SH-SY5Y cells with a half lethal concentration (LC_{50}) ~10X lower and rat PC-12 cells (with a LC_{50} ~2X lower) (52). Rotenone is prevalently utilized as a trigger to induce neurodegenerative alterations in both *in vitro* and *in vivo* experimental models of PD (56,57). Intracellular calcium, rather than oxidative stress, constitutes a major factor for rotenone-induced apoptosis in neuronal cells (58).

A previous study has found that the cell viability of N2A cells is decreased after treatment with 10 nmol/l deguelin for 24-72 h in a time-dependant manner or after the treatment of 0.01-1 μmol/l deguelin for 48 h in a dose-dependant manner, with IC_{50} of 16 nmol/l (59). Rotenine is a structural analogue of degulin in terms of its chemical composition (60). The cell survival rate was found to be 1.000±0.039 in the control group and decreased by 65.8% to 0.342±0.042 after rotenone (20 nmol/l) treatment. Elevated concentrations of rotenone resulted in increased cell death (59). After the pre-experiment of cell viability, the concentration of rotenone (20 nmol/l) was established in the present cellular experiments. Significant inhibition of mitochondrial complex I activities by 5 μmol/l rotenone in SK-N-MC cells was reported by Sherer *et al* (61). In the experiment of the mitochondrial complex I assay, 2.5 μmol/l rotenone was introduced to isolated mitochondria instead of to N2A cells. In all the experiments apart from the mitochondrial complex I assay, 20 nmol/l rotenone was administered to N2A cells. The concentration of rotenone utilized in the mitochondrial complex I assay was significantly higher compared with that employed in the other assays. This discrepancy arises from the fact that, in the mitochondrial complex I assay, rotenone acted on the mitochondria isolated from N2A cells for 10 min. By contrast, during the other experiments, rotenone continued to exert its effects on N2A cells over a duration of 48 h.

The secondary root extract of HP is rich in bioactive iridoid glycosides, specifically known as harpagoside (62). Extracts of HP have demonstrated a concentration-dependent inhibition of lipid peroxidation in brain homogenates induced by different pro-oxidants (Fe^{2+} or sodium nitroprusside) (63). In addition, the ethyl acetate fraction of HP exhibited the most pronounced antioxidant effects by either reducing lipid peroxidation and cellular damage or restoring thiol levels and catalase activity in brain cortical slices induced by different pro-oxidants (63). HP extracts have also been reported to upregulate brain-derived neurotrophic factor gene expression and downregulating TNF-α gene expression in rat cortex synaptosomes treated with amyloid β-peptide (64). Furthermore, the extracts mitigated amyloid β-peptide-induced stimulation of malondialdehyde and 3-hydroxykynurenine levels and attenuated the reduction in DA, norepinephrine and serotonin concentrations in the rat cortex treated with amyloid β-peptide (64). Harpagoside was demonstrated to be effective in providing promotion of axonal outgrowth in primary spinal cord neurons under reactive oxygen species-insulting conditions induced by ferrous sulfate (65). In addition, previous studies have found that harpagoside (0.1-10 μmol/l) isolated from *Scrophulariae* can protect against glutamate-induced cortical neuronal injury in rats (66), improve memory in mice injured by scopolamine and exhibit antioxidant activity (67). According to Li *et al* (68), harpagoside alleviated amyloid-β-induced cognitive disorders in rats by upregulating brain-derived neurotrophic factor expression and the MAPK/PI3K signaling pathway. Harpagoside reduced neuroinflammation-induced neuronal damage by inhibiting the Toll-like receptor 4/molecule myeloid differentiation factor 88/NF-κB pathway (69,70). In another study, harpagoside suppressed the overactivation of PTEN induced by chronic cerebral hypoperfusion. Specifically, harpagoside increased the activity of Akt and inhibited the activity of GSK-3β, a downstream effector of PTEN (31). Sun *et al* (32) previously found that harpagoside can alleviate the symptoms of MPTP/MPP^+-induced DA neurodegenerative changes and movement disorders by increasing GDNF mRNA and GDNF protein expession levels. Li *et al* (36) found that harpagoside protected against doxorubicin-induced cardiotoxicity through p53/Parkin-mediated mitophagy. Harpagoside influences the p53/Parkin-mediated cascade involving mitophagy deficiency, mitochondrial dyshomeostasis and apoptosis through a novel interaction between p53 and Parkin (36), whereby harpagoside promoted Parkin translocation to mitochondria and substantially restored Parkin-mediated mitophagy by inhibiting the binding of p53 and Parkin. In the present study, harpagoside (10 μmol/l) exerted a significant protective effect on rotenone-induced N2A cells. When the harpagoside concentrations were ≥0.1 μmol/l, they significantly prevented rotenone-induced mitochondrial damage in N2A cells and relieved rotenone-induced mitochondrial swelling. However, harpagoside (1 μmol/l) significantly antagonized the rotenone-induced inhibition of complex I. The harpagoside (10 μmol/l) significantly inhibited rotenone-induced caspase 3 activation.

To conclude, results from the present study suggests that harpagoside can protect against a rotenone-induced pseudo-PD cell model through the mitochondrial protection pathway. However, it must be noted that experiments in the present study were performed using spectrophotometry, without support using other research methods or further delving into the impact on other genes/proteins, including cytochrome *c*, pro-apoptotic genes/proteins and caspase 9. In future studies, experimental investigations into mitochondrial-related signaling pathways should be performed by incorporating additional detection methods, such as WB. In addition, whether harpagoside can regulate the mitochondrial pathway through its influence on oxidative stress and determine whether harpagoside can exert an impact on mitochondria-dependent apoptotic signaling should be assessed.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

ZX was the main contributor to writing the manuscript. JL and ZX designed the experiments and were responsible for the statistical and data analysis. JL and ZX drafted and revised the original manuscript. JL and ZX confirmed the authenticity of all original data and interpreted the results of the study and gave the final approval for the forthcoming version. JL and ZX read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Tolosa E, Garrido A, Scholz SW and Poewe W: Challenges in the diagnosis of Parkinson's disease. Lancet Neurol 20: 385-397, 2021.
2. Zhou ZD, Yi LX, Wang DQ, Lim TM and Tan EK: Role of dopamine in the pathophysiology of Parkinson's disease. Transl Neurodegener 12: 44, 2023.
3. Norel R, Agurto C, Heisig S, Rice JJ, Zhang H, Ostrand R, Wacnik PW, Ho BK, Ramos VL and Cecchi GA: Speech-based characterization of dopamine replacement therapy in people with Parkinson's disease. NPJ Parkinsons Dis 6: 12, 2020.
4. Yang X, Wang J, Zeng W, Zhang X, Yang X, Xu Y, Xu Y and Cao X: Time-dependent alterations in the rat nigrostriatal system after intrastriatal injection of fibrils formed by α-Syn and tau fragments. Front Aging Neurosci 14: 1049418, 2022.
5. Sosnik R, Fahoum F, Katzir Z, Mirelman A and Maidan I: Key shifts in frontoparietal network activity in Parkinson's disease. NPJ Parkinsons Dis 11: 2, 2025.
6. Schapira AH: Mitochondria in the aetiology and pathogenesis of Parkinson's disease. Lancet Neurol 7: 97-109, 2008.
7. Schapira AH, Gu M, Taanman JW, Tabrizi SJ, Seaton T, Cleeter M and Cooper JM: Mitochondria in the etiology and pathogenesis of Parkinson's disease. Ann Neurol 44 (3 Suppl 1): S89-S98, 1998.
8. Ondo W: IPX066, a mixed immediate/sustained-release levodopa preparation for Parkinson's disease. Expert Opin Pharmacother 15: 2081-2085, 2014.
9. Caronni S, Del Sorbo F, Barichella M, Fothergill-Misbah N, Denne T, Laguna J, Urasa S, Dekker MCJ, Akpalu A, Sarfo FS, *et al*: Mucuna pruriens to treat Parkinson's disease in low-income countries: Recommendations and practical guidelines from the farmer to clinical trials. Paving the way for future use in clinical practice. Parkinsonism Relat Disord 124: 106983, 2024.
10. Kumari S, Gupta S, Sukhija R, Gurjar S, Dubey SK and Taliyan R: Neuroprotective potential of Epigenetic modulators, its regulation and therapeutic approaches for the management of Parkinson's disease. Eur J Pharmacol 985: 177123, 2024.
11. Guo F, Qin X, Mao J, Xu Y and Xie J: Potential protective effects of pungent flavor components in neurodegenerative diseases. Molecules 29: 5700, 2024.
12. Filaferro M, Avallone R, Rustichelli C and Vitale G: Characterization of walnut oil and evaluation of its neuroprotective effects in an in vitro model of Parkinson's disease. Molecules 29: 5718, 2024.
13. Kim JH, Huh E, Eo H, Kim JS, Kwon Y, Ju IG, Choi Y, Yoon HJ, Son SR, Jang DS, *et al*: Tribuli Fructus alleviates 1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine (MPTP)-induced Parkinson's disease by suppressing neuroinflammation via JNK signaling. Metab Brain Dis 40: 69, 2024.
14. Natarajan K, Chandrasekaran R, Sundararaj R, Joseph J and Asaithambi K: Neuroprotective assessment of nutraceutical (Betanin) in neuroblastoma cell line SHSY-5Y: An in-vitro and in-silico approach. Neurochem Res 50: 54, 2024.
15. Hvingelby VS and Pavese N: Surgical advances in Parkinson's disease. Curr Neuropharmacol 22: 1033-1046, 2024.
16. Rahimi Darehbagh R, Seyedoshohadaei SA, Ramezani R and Rezaei N: Stem cell therapies for neurological disorders: Current progress, challenges, and future perspectives. Eur J Med Res 29: 386, 2024.
17. Huang S, Liu H, Lin Y, Liu M, Li Y, Mao H, Zhang Z, Zhang Y, Ye P, Ding L, *et al*: Berberine protects against NLRP3 inflammasome via ameliorating autophagic impairment in MPTP-induced Parkinson's disease model. Front Pharmacol 11: 618787, 2020.
18. Vellingiri B, Chandrasekhar M, Sri Sabari S, Gopalakrishnan AV, Narayanasamy A, Venkatesan D, Iyer M, Kesari K and Dey A: Neurotoxicity of pesticides-A link to neurodegeneration. Ecotoxicol Environ Saf 243: 113972, 2022.
19. Johnson ME and Bobrovskaya L: An update on the rotenone models of Parkinson's disease: Their ability to reproduce the features of clinical disease and model gene-environment interactions. Neurotoxicology 46: 101-116, 2015.
20. Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV and Greenamyre JT: Chronic systemic pesticide exposure reproduces features of Parkinson's disease. Nat Neurosci 3: 1301-1306, 2000.
21. Tao X, Zhang W, Chen C, Tao Y, Tao Y, Chen Z and Zhang G: miR-101a-3p/ROCK2 axis regulates neuronal injury in Parkinson's disease models. Aging (Albany NY) 16: 8732-8746, 2024.
22. Menchinskaya E, Chingizova E, Pislyagin E, Likhatskaya G, Sabutski Y, Pelageev D, Polonik S and Aminin D: Neuroprotective effect of 1,4-naphthoquinones in an in vitro model of paraquat and 6-OHDA-induced neurotoxicity. Int J Mol Sci 22: 9933, 2021.

23. Verma A and Goyal A: Plumbagin's healing effect on motor impairment in rotenone-toxified rodents. Curr Neurovasc Res: Sep 3, 2024 (Epub ahead of print).
24. Olubodun-Obadun TG, Ishola IO, Folarin OR, Oladoja FA, Gilbert TT, Aniekwensi IM, Bisiriyu A, Joseph-Iwebi NA, Adebanjo FO, Olopade JO and Adeyemi OO: Cajanus cajan (L) Millsp seeds extract prevents rotenone-induced motor- and non-motor features of Parkinson disease in mice: Insight into mechanisms of neuroprotection. J Ethnopharmacol 322: 117623, 2024.
25. Meng X, Xie W, Xu Q, Liang T, Xu X, Sun G and Sun X: Neuroprotective effects of radix scrophulariae on cerebral ischemia and reperfusion injury via MAPK pathways. Molecules 23: 2401, 2018.
26. Lee HJ, Spandidos DA, Tsatsakis A, Margina D, Izotov BN and Yang SH: Neuroprotective effects of Scrophularia buergeriana extract against glutamate-induced toxicity in SH-SY5Y cells. Int J Mol Sci 43: 2144-2152, 2019.
27. Kim HL, Min DE, Lee SK, Choi BK and Lee DR: Scrophularia buergeriana Extract (Brainon) attenuates neuroinflammation in BV-2 microglia cells and promotes neuroprotection in SH-SY5Y neuroblastoma cells. J Med Food 26: 328-341, 2023.
28. Azadmehr A, Oghyanous KA, Hajiaghaee R, Amirghofran Z and Azadbakht M: Antioxidant and neuroprotective effects of Scrophularia striata extract against oxidative stress-induced neurotoxicity. Cell Mol Neurobiol 33: 1135-1141, 2013.
29. Salavati P, Ramezani M, Monsef-Esfahani HR, Hajiagha R, Parsa M, Tavajohi S and Ostad SN: Neuroprotective effect of total and sequential extract of scrophularia striata boiss. in rat cerebellar granule neurons following glutamate-induced neurotoxicity: An in-vitro study. Iran J Pharm Res 12: 389-394, 2013.
30. Wang K, Lou Y, Xu H, Zhong X and Huang Z: Harpagide from Scrophularia protects rat cortical neurons from oxygen-glucose deprivation and reoxygenation-induced injury by decreasing endoplasmic reticulum stress. J Ethnopharmacol 253: 112614, 2020.
31. Chen C, Zhang H, Xu H, Xue R, Zheng Y, Wu T and Lian Y: Harpagoside rescues the memory impairments in chronic cerebral hypoperfusion rats by inhibiting PTEN activity. J Alzheimers Dis 63: 445-455, 2018.
32. Sun X, Xiong Z, Zhang Y, Meng Y, Xu G, Xia Z, Li J, Zhang R, Ke Z, Xia Z and Hu Y: Harpagoside attenuates MPTP/MPP(+) induced dopaminergic neurodegeneration and movement disorder via elevating glial cell line-derived neurotrophic factor. J Neurochem 120: 1072-1083, 2012.
33. Dinda B, Dinda M, Kulsi G, Chakraborty A and Dinda S: Therapeutic potentials of plant iridoids in Alzheimer's and Parkinson's diseases: A review. Eur J Med Chem 169: 185-199, 2019.
34. Wang Y, Shinoda Y, Cheng A, Kawahata I and Fukunaga K: Epidermal fatty acid-binding protein 5 (FABP5) involvement in alpha-synuclein-induced mitochondrial injury under oxidative stress. Biomedicines 9: 110, 2021.
35. He L, Wang J, Yang Y, Li J and Tu H: Mitochondrial sirtuins in Parkinson's disease. Neurochem Res 47: 1491-1502, 2022.
36. Li W, Wang X, Liu T, Zhang Q, Cao J, Jiang Y, Sun Q, Li C, Wang W and Wang Y: Harpagoside protects against doxorubicin-induced cardiotoxicity via P53-parkin-mediated mitophagy. Front Cell Dev Biol 10: 813370, 2022.
37. Tremblay RG, Sikorska M, Sandhu JK, Lanthier P, Ribecco-Lutkiewicz M and Bani-Yaghoub M: Differentiation of mouse Neuro 2A cells into dopamine neurons. J Neurosci Methods 186: 60-67, 2010.
38. Hergenhahn L, Padutsch N, Azawi S, Weiskirchen R, Liehr T and Rincic M: Cytogenomic characterization of murine neuroblastoma cell line neuro-2a and its two derivatives neuro-2a TR-alpha and Neuro-2a TR-Beta. Cells 13: 1889, 2024.
39. Lei C, Wang J, Zhang X, Ge X, Zhao W, Li X, Jiang W, Ma M, Wang Z, Sun S, *et al*: The wnt/pyruvate kinase, muscle axis plays an essential role in the differentiation of mouse neuroblastoma cells. Neurochem Int 181: 105901, 2024.
40. Kumar M and Katyal A: Data on retinoic acid and reduced serum concentration induced differentiation of Neuro-2a neuroblastoma cells. Data Brief 21: 2435-2440, 2018.
41. Subramaniam SR and Chesselet MF: Mitochondrial dysfunction and oxidative stress in Parkinson's disease. Prog Neurobiol 106-107: 17-32, 2013.
42. Chen R, Gu X and Wang X: α-Synuclein in Parkinson's disease and advances in detection. Clin Chim Acta 529: 76-86, 2022.
43. Bose A and Beal MF: Mitochondrial dysfunction in Parkinson's disease. J Neurochem 139: 216-231, 2016.
44. Perez MJ, Baden P and Deleidi M: Progresses in both basic research and clinical trials of NAD+ in Parkinson's disease. Mech Ageing Dev 197: 111499, 2021.
45. Yang Y and Lu B: Mitochondrial morphogenesis, distribution, and Parkinson disease: Insights from PINK1. J Neuropathol Exp Neurol 68: 953-963, 2009.
46. Vila M, Ramonet D and Perier C: Mitochondrial alterations in Parkinson's disease: new clues. J Neurochem 107: 317-328, 2008.
47. Casoli T, Lisa R, Fabbietti P and Conti F: Analysis of mitochondrial DNA allelic changes in Parkinson's disease: A preliminary study. Aging Clin Exp Res 32: 345-349, 2020.
48. Henchcliffe C and Beal MF: Mitochondrial biology and oxidative stress in Parkinson disease pathogenesis. Nat Clin Pract Neurol 4: 600-609, 2008.
49. Shen L and Dettmer U: Alpha-synuclein effects on mitochondrial quality control in Parkinson's disease. Biomolecules 14: 1649, 2024.
50. Yang JL, Weissman L, Bohr VA and Mattson MP: Mitochondrial DNA damage and repair in neurodegenerative disorders. DNA Repair (Amst) 7: 1110-1120, 2008.
51. Pathak RU and Davey GP: Complex I and energy thresholds in the brain. Biochim Biophys Acta 1777: 777-782, 2008.
52. Mazzio EA, Soliman YI and Soliman KF: Variable toxicological response to the loss of OXPHOS through 1-methyl-4-phenylpyridinium-induced mitochondrial damage and anoxia in diverse neural immortal cell lines. Cell Biol Toxicol 26: 527-539, 2010.
53. Wong CK, Yeung HY, Mak NK, DiMattia GE, Chan DK and Wagner GF: Effects of dibutyryl cAMP on stanniocalcin and stanniocalcin-related protein mRNA expression in neuroblastoma cells. J Endocrinol 173: 199-209, 2002.
54. Ferrari E, Cardinale A, Picconi B and Gardoni F: From cell lines to pluripotent stem cells for modelling Parkinson's disease. J Neurosci Methods 340: 108741, 2020.
55. LePage KT, Dickey RW, Gerwick WH, Jester EL and Murray TF: On the use of neuro-2a neuroblastoma cells versus intact neurons in primary culture for neurotoxicity studies. Crit Rev Neurobiol 17: 27-50, 2005.
56. Agafonova I, Chingizova E, Chaikina E, Menchinskaya E, Kozlovskiy S, Likhatskaya G, Sabutski Y, Polonik S, Aminin D and Pislyagin E: Protection activity of 1,4-naphthoquinones in rotenone-induced models of neurotoxicity. Mar Drugs 22: 62, 2024.
57. Xia N, Madore V, Albalakhi A, Lin S, Stimpson T, Xu Y, Schwarzschild MA and Bakshi R: Microglia-dependent neuroprotective effects of 4-octyl itaconate against rotenone-and MPP+-induced neurotoxicity in Parkinson's disease. Sci Rep 13: 15539, 2023.
58. Swarnkar S, Goswami P, Kamat PK, Gupta S, Patro IK, Singh S and Nath C: Rotenone-induced apoptosis and role of calcium: A study on Neuro-2a cells. Arch Toxicol 86: 1387-1397, 2012.
59. Xiong Z Wang S and Lang J: Inhibitory effects of deguelin on the viability of neuro-2A cells. Chin Pharmacist 17: 1793-1796, 2014 (In Chinese).
60. Caboni P, Sherer TB, Zhang N, Taylor G, Na HM, Greenamyre JT and Casida JE: Rotenone, deguelin, their metabolites, and the rat model of Parkinson's disease. Chem Res Toxicol 17: 1540-1548, 2004.
61. Sherer TB, Betarbet R, Testa CM, Seo BB, Richardson JR, Kim JH, Miller GW, Yagi T, Matsuno-Yagi A and Greenamyre JT: Mechanism of toxicity in rotenone models of Parkinson's disease. J Neurosci 23: 10756-10764, 2003.
62. Kondamudi N, Turner MW and McDougal OM: Harpagoside content in devil's claw extracts. Nat Prod Commun 11: 1215-1216, 2016.
63. Schaffer LF, Peroza LR, Boligon AA, Athayde ML, Alves SH, Fachinetto R and Wagner C: Harpagophytum procumbens prevents oxidative stress and loss of cell viability in vitro. Neurochem Res 38: 2256-2267, 2013.
64. Ferrante C, Recinella L, Locatelli M, Guglielmi P, Secci D, Leporini L, Chiavaroli A, Leone S, Martinotti S, Brunetti L, *et al*: Protective effects induced by microwave-assisted aqueous harpagophytum extract on rat cortex synaptosomes challenged with amyloid β-peptide. Phytother Res 31: 1257-1264, 2017.
65. Hong JY, Kim H, Yeo C, Lee J, Jeon WJ, Lee YJ and Ha IH: Epidural injection of harpagoside for the recovery of rats with lumbar spinal stenosis. Cells 12: 2281, 2023.
66. Kim SR, Lee KY, Koo KA, Sung SH, Lee NG, Kim J and Kim YC: Four new neuroprotective iridoid glycosides from Scrophularia buergeriana roots. J Nat Prod 65: 1696-1699, 2002.

67. Jeong EJ, Lee KY, Kim SH, Sung SH and Kim YC: Cognitive-enhancing and antioxidant activities of iridoid glycosides from Scrophularia buergeriana in scopolamine-treated mice. Eur J Pharmacol 588: 78-84, 2008.
68. Li J, Ding X, Zhang R, Jiang W, Sun X, Xia Z, Wang X, Wu E, Zhang Y and Hu Y: Harpagoside ameliorates the amyloid-β-induced cognitive impairment in rats via up-regulating BDNF expression and MAPK/PI3K pathways. Neuroscience 303: 103-114, 2015.
69. Hao RJ, Yu GR, Qin XD and Lu YW: Harpagoside alleviates neuronal damage caused by neuroinflammation via suppressing TLR4/MyD88/NF-κB pathway. Chin Tradit Herbal Drugs 54: 4202-4213, 2023 (In Chinese).
70. Huang TH, Tran VH, Duke RK, Tan S, Chrubasik S, Roufogalis BD and Duke CC: Harpagoside suppresses lipopolysaccharide-induced iNOS and COX-2 expression through inhibition of NF-kappa B activation. J Ethnopharmacol 104: 149-155, 2006.

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