Compound Dihuang Granule Attenuates the DA Neuron Loss and Motor Deficits in 6-OHDA Induced Parkinson's Disease Rats

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Research

Keywords: Parkinson's disease, Compound Dihuang Granule, Oxidative stress, Apoptosis, DA neurons

Posted Date: September 24th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-81379/v1>

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Abstract

Background: Parkinson's disease (PD) is a multifactorial neurodegenerative disorder characterized by progressive loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) and the presence of Lewy bodies (LBs) consisting of misfolded α -synuclein protein in the substantia nigra pars compacta (SNpc). Compound Dihuang Granule (CDG), a famous traditional Chinese medicine (TCM) has been clinically used in PD therapy with curative effects. However, the specific functions and the mechanism of action remained unclear. This paper study assesses the preventive and therapeutic effect of CDG on motor deficits and DA neuron loss of PD induced by 6-OHDA and the underlying mechanisms.

Methods: PD rat model was induced by unilaterally stereotactic injection of 6-OHDA into the SNpc of midbrain then the motor deficits were evaluated with apomorphine (APO) induced abnormal rotational behaviors. The striatal contents of neurotransmitters were detected by high performance liquid chromatography with electrochemical detection (HPLC-ECD). The number of DA neurons were determined with immunohistochemistry (IHC) staining. Protein expression levels were determined with Western blotting assay. Indicators of oxidative stress were determined with colorimetric method. Apoptotic cells were detected by Transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) assay. The expression of neurotrophic factors was examined with IHC staining.

Results: With a 6-week treatment, CDG significantly attenuated the 6-OHDA induced abnormal rotational behaviors and alleviated the loss of DA neurons in the nigrostriatal axis. Consistently, the striatal contents of DA and its metabolites including DOPAC and HVA of PD rats were all significantly increased with CDG treatment. The 6-OHDA induced oxidative stress indicated with decreased superoxide dismutase (SOD), glutathione (GSH), glutathione peroxidase (GSH-Px) and increased malondialdehyde (MDA) was also suppressed by CDG. Moreover, CDG treatment inhibited the 6-OHDA induced cell apoptosis indicated with decreased apoptotic cells in the SNpc and increased protein expression ratio of Bcl-2/Bax in the striatum. The expression levels of neurotrophic factors including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and glial-derived neurotrophic factor (GDNF) in the SNpc of PD rats were also increased by CDG.

Conclusion: CDG could ameliorate the 6-OHDA induced brain injuries and motor symptoms mainly by inhibition of the oxidative stress and cell apoptosis in the nigrostriatal axis, and enhancing the expression of neurotrophic factors in the midbrain of rats.

Background

Parkinson's disease (PD) is the second most common neurodegenerative disease with 2% of people aged over 60 years old suffering all over the world. PD is clinically manifested with static tremor, bradykinesia, rigidity and abnormal posture [1], while pathologically characterized with progressive loss of DA neurons and deposition of α -synuclein-containing LBs in the SNpc [2]. The etiology and pathogenesis of PD is complicated and remains unclear. So far, no treatment is available to effectively slow down or halt PD

progression [3]. Levodopa is the only available treatment reported to extend life expectancy [4]. However, as long-term usage of the drug, the therapeutic effects become increasingly less beneficial, and more than 50% of patients eventually experience highly disabling fluctuations, dyskinesia and agonist induced sleep attacks [5-7]. Therefore, it is imminent to find alternative therapy with less toxic side effects.

A variety of intracellular processes are involved in the pathogenesis of PD, including mitochondrial dysfunction, oxidative stress, cell apoptosis and neurotrophic factors deprivation. Oxidative stress plays an undeniable role in a complex and progressive neurodegenerative cascade, the inhibition of which attenuates DA neuron loss in PD models [8,9]. Apoptosis has been implicated as the main mechanism of neuronal death in PD. Apoptosis is mediated by a number of initiator and executioner caspases, and occurs via the intrinsic or extrinsic pathways [10]. Amounts of Chinese herb extracts delivered protective effects by suppressing cell apoptosis and oxidative stress [11-13]. Neurotrophic factors are endogenous proteins promoting the survival of different neural cells, thus eliciting great interest as a possible treatment for neurodegenerative disorders [14]. Upregulation of neurotrophic factors has been an effective approach for physical and medical treatments to protect against neurotoxicity induced neurodegeneration in PD [15,16]. Therefore, targeting the multiple pathological prospects of PD could turn to effective approach in clinical treatments.

Traditional Chinese medicine is famous for the multidimensional clinical outcome in medical treatments. CDG, a classical prescription, has been clinically used in PD therapy for improving motor and non-motor symptoms and reducing the side effects of long-term Levodopa usage. CDG consists of *Rehmanniae radix praeparata* (SDH), *Paeoniae radix alba* (BS), *Uncariae ramulus cum uncis* (GT), *Margaritifera concha* (ZZM), *Salviae miltiorrhizae radix et rhizome* (DS), *Acori tatarinowii rhizome* and *Scorpio* (QX) (table1). In our previous study, CDG was proved to alleviate the excess levodopa induced dyskinesia in PD rat model [17]. In CDG, Dihuang (*Rehmanniae radix praeparata*) is considered as the sovereign drug, which plays the most vital role in the whole formula. A number of studies indicate that multiple components like iridoid glycosides including catalpol and verbascoside separated from DiHuang protect against neurodegeneration of PD. Catalpol alleviates MPTP-triggered oxidative stress and thereby prevents neurodegenerative diseases-related inflammatory reaction [18]. Verbascoside, an extract from *Radix Rehmanniae Praeparata* has been shown to be effective in treating PD and can increase the TH content of PD rats [19]. The other active ingredients of the monomers of CDG also showed protective effects in PD models. The paeoniflorin and paeonol derived from *Paeoniae radix alba* had neuroprotective effects in an in vitro model of PD [20]. *Acorus gramineus* extract exerted anti-neuroinflammatory effects against activated microglia mediated insults through multiple signaling pathways and prevented in vivo neuronal cell death in mouse model of PD [21]. Tanshinone I treatment significantly attenuated 6-OHDA-induced striatal oxidative stress and ameliorated dopaminergic neurotoxicity in 6-OHDA-lesioned mice [22].

Therefore, these studies suggested that a protective role of CDG against neuronal injuries in PD. However, the protective effects and specific mechanisms of CDG in PD remain to be further investigated. In this

study, the beneficial effects of CDG will be documented with 6-OHDA induced PD rats and the mechanisms will be investigated.

Methods

Chemical reagents

6-OHDA, Apomorphine, DA and DOPAC were purchased from Sigma Chemicals (St. Louis, MO, USA). Madopar was purchased from Shanghai Roche Pharmaceuticals Ltd (Shanghai, China). HVA was purchased from TCI (Shanghai) Chemical Industry Co., Ltd (Shanghai, China). Nanjing Institute of biological engineering kit was purchased from Nanjing Jiancheng Biological Engineering Research Institute Co., Ltd (Nanjing, China). In situ cell death detection kit was purchased from Roche Molecular Systems, Inc (Basel, Switzerland). All other chemicals were commercially available and of reagent grade.

Preparation of CDG

CDG was manufactured by Shanghai Traditional Chinese Medicine Pharmaceutical Technology Co., Ltd (Shanghai, China) (lot number, 20140102), which composed of SDH, BS, GT, ZZM, DS, SCP and QX in a dry weight ratio of 20:30:15:15:20:12:2 (table 1). Herbs mixtured as the required proportion were cut into slices and soaked in distilled water for 30 minutes, then boiled twice with 10 times and 8 times volume of distilled water respectively, 1 h each. The extracts were merged and filtered, then concentrated into the cream form with the relative density of 1.30. The semi-manufactures obtained were dried, sifted and then powdered to generate CDG. The electron spray ionization mass spectrometry (ESI-MS) analysis of CDG was observed in Supplementary Material. The performing standard of CDG used in the present study was in line with Chinese Pharmacopeia (2010 version). In the animal experiment, rats daily dosage of CDG was converted from human daily dosage with the equation $DB\text{ (rat)} = DA\text{ (human)} \times 7/388$. Thus 6.3 times of the normal dosage for adult human was defined as the dosage of CDG for rats. Rats in CDG groups were given 7g/kg/day of CDG dissolving in distilled water at a concentration of 0.7g/ml.

Experimental Animals

Male Sprague-Dawley rats, weighing 160-200g, were provided by the Animal Experimental Center of Shanghai University of TCM, China (license No. SYXK (Hu) 2020-0009). Rats were housed in wire cages at $23 \pm 2^\circ\text{C}$ and 60-65% humidity, with illumination of 12-hour dark/light cycle (light 7:00-19:00, dark 19:00-7:00), with access to water and food ad libitum. All experimental procedures were conducted according to the National Institute of Health Guide for the Care and Use of Laboratory Animals, and approved by the Animal Care Committee of Shanghai University of TCM.

6-OHDA induced PD model

The rats were anesthetized with 3% pentobarbital sodium (50mg/kg; intraperitoneal) and were fixed on a stereotaxic apparatus (Shanghai Leica Instruments Co., Ltd., Shanghai, China). 6-OHDA (diluted in normal saline containing 0.2% ascorbic acid; Sigma, St. Louis, MO, USA), or sterile saline (3 μL at each point) were

injected at two points in the SNpc (Bregma coordinates for two injection points-first point:5.2 mm posterior to the bregma, 1.0 mm right lateral to the midline, 9.0 mm below the dura mater; and second point:5.2 mm posterior to the bregma, 2.5 mm right lateral to the midline, 8.5 mm below the dura mater) at the rate of 1 μ L/min by 5 μ L Hamilton syringe. After the injection, the needle was retained in SNpc for 5 min. Then the needle was withdrawn at the rate of 1 mm/min. After surgery, the rats were returned to the same environment where they were in prior to surgery. Two weeks after the operation, the rats' contralateral rotations induced by Apomorphine (APO) were measured and recorded with a video camera at 2 weeks,4 weeks and 6 weeks. The duration of each recording time was 30 min. Rats with a rotating frequency of over 7 turns per minute were included in the PD model.

Animal Treatment

Rats were randomly divided into Sham-operated group (n = 10) and model-operated group (n = 80). Successful rat models of PD were randomly divided into an untreated PD group (n = 10), a Madopar-treated PD group (n = 10) and a CDG-treated PD group (n = 10). Rats in the CDG group were fed with 7g/kg CDG, rats in Madopar group were gavaged with 150mg/kg Madopar and other groups were given the same amount of saline. Each gavage volume was 1mL/100g, once a day for 6 weeks.

Collection of Tissue Sample

Rats were all sacrificed then the fresh tissues of SNpc and striatum were quickly harvested. The samples were collected on the ice box and placed in a refrigerator at -80°C for subsequent detection of relevant indicators. For immunohistochemistry and immunofluorescence experiments, the whole rat brain was immersed in 10% neutral formaldehyde. Brains were serially cut into coronal sections at a thickness of 20 μ m by using frozen microtome (Leica, Germany) and stored at -20°C in a cryoprotectant solution for further histologic analysis. DA neurons in SNpc were detected by DAB staining, for the detection of nutritional factors in this experiment, we used paraffin embedded and sliced, the thickness is 3.5 μ m.

Rotation test

Rats were intraperitoneally injected with 0.5mg/kg APO to induce contralateral rotational behaviors. The test was recorded for 30 minutes and the number of rotations were scored.

High performance liquid chromatography (HPLC)

After the behavioral test, the striatum was homogenized in 0.4 mol·L⁻¹ perchloric acid solution (1mg: 40 μ L). After centrifugation at 12000 r/min for 15 min at 4°C, the supernatant was collected and placed on ice until further HPLC analysis. The supernatant was used to measure the concentrations of DA and its metabolites DOPAC and HVA by HPLC with electrochemical detection (EC), as previously described [23]. The chromatographic conditions were as follows: the Welch XB-C18 column (4.6×250mm,5 μ m); mobile phase: 6% methanol, 0.035mol·L⁻¹ anhydrous citric acid,0.09mol·L⁻¹ sodium acetate anhydrous,

0.23mmol·L⁻¹ sodium octyl sulfonate, 0.13mmol·L⁻¹ EDTA. and pH=4.1; flow rate: 1 ml/min; and injection volume: 50μL.

Western blotting analysis

The striatum tissues were lysed in T-PERTM Tissue Protein Extraction Reagent (Thermo Scientific, USA) containing complete protease inhibitor. Then, the protein extracts were centrifuged for 30 min at 12000×g, and the supernatant was collected. Protein concentrations were measured using a BCA kit (Beyotime biotechnology Inc., Shanghai, China). The protein supernatant was denatured in loading buffer and boiled in water for 10 min, and the protein samples were stored at -20 °C. Then, 40μg of protein from each group was separated by 10% SDS-PAGE gels and electrophoresis and subsequently transferred onto a PVDF membrane (0.45μm, EMD Millipore, Billerica, MA, USA). BSA (3%; Sigma-Aldrich, St. Louis, MO, USA) was used to block the membranes for 2 h at room temperature (RT). The membranes were then incubated with primary antibody TH (1:1000 dilution, Cell Signaling Technology, CST Inc., MA, USA), Bax (1:200 dilution; 2772s, CST Inc.), Bcl-2 (1:500 dilution; 2676s, CST Inc.) overnight at 4 °C. After the membranes were washed three times with tris-buffered saline containing 0.1% Tween-20 (TBST), they were incubated with the horseradish peroxidase-conjugated antibody mouse β-Actin (1:3000 dilution; Santa Cruz Biotechnology, Inc., Dallas, USA), GAPDH Antibody Mouse Monoclonal (1:3000 dilution; Proteintech Group, Inc, Rosemont, USA) for 1 h at RT. After the final wash, signals were detected using Li-cor ODDSEY infrared laser imaging system (CLx-1259, LI-COR Biosciences, USA); Image J software was used to analyze the strip optical density.

Immunohistochemistry (IHC)

For IHC, the 20-μm-thick slices of rat brain tissue from each group were selected as similar as possible. And frozen slices were subjected to citrate buffer (0.1 M, pH 6.0) at 95 °C for 10 min for antigen retrieval. After the tissue was washed three times with phosphate-buffered saline containing 0.2% Tween-20 (PBST) for 10 min, the sections were treated with 0.5% Triton X-100 for 10 min and blocked with 5% bovine serum albumin (BSA) for 1 h at RT. After blocking, the sections were incubated with anti- mouse TH (1:1000 dilution; ab112, Cambridge, MA, USA), which was prepared with PBST (0.5% Triton X-100) /1% sheep serum, incubated at 37 °C for 2 h, 4 °C overnight. Then, samples were incubated with the horseradish peroxidase-conjugated secondary antibody (1:200 dilution; Abcam, Cambridge, MA, USA) for 1 h, and samples were detected with 3,3'-diaminobenzidine (DAB) for 2-3 min. Finally, the sections were cover-slipped with neutral balsam and observed with an Olympus BA51 photomicroscope (Tokyo, Japan). Image Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA) was used for cell counting.

For the detection of neurotrophic factors, the 3.5-μm-thick slices were mounted on glass slides and baked for 1h at 62 °C, after which they were deparaffinized and the endogenous peroxidase activity quenched. The primary antibody NGF (1:200 dilution; Abcam, ab52918), BDNF (1:400 dilution; Abcam, ab108319), GDNF (1:400 dilution; Abcam, ab176564) were incubated on the slides for 12 h at 4 °C. After rinsing three times with phosphate-buffered saline solution containing Tween, the horseradish peroxidase-conjugated

secondary antibody (1:200 dilution; Abcam, Cambridge, MA, USA) was incubated for 20 min at RT and then visualized after incubation with 3, 3-diaminobenzidine for 10 min at RT. Then, the sections were counterstained with hematoxylin to mark the nucleus. Finally the binding sites were sealed with neutral resin. Images were obtained at the objective len with 20×magnification. The number of positive cells were counted by Image J software.

Immunofluorescence staining

Brain frozen slices with 20μm thick from each group were washed with PBS five times for 3 min each. Then, 0.5% (wt/vol) Triton X-100 and blocking serum were added successively and incubated for 10 min and 1.5 h, respectively. The tissue was incubated in primary antibody, anti- mouse TH (1:1000 dilution; ab112, Abcam Inc., Cambridge, USA), at 4 °C overnight. After being washed four times with PBS, the sections were incubated with the secondary antibody (1:1000 dilution; A27034, Alexa Fluor 488; A-11007, Alexa Fluor 555; Invitrogen, Carlsbad, CA, USA) for 1h at RT and protected from light. Images were obtained at 5× and 20× the original magnification. The relative area immunoreactivity was calculated with Image J software.

Measurement of oxidative stress level

Rats from each group were anesthetized with pentobarbital sodium (50mg/kg), decapitated and their brains removed, then the right substantia nigra was dissected out and weighed.(1) removal of the brain 0.5g in the cold saline to remove blood, rinse, dry filter paper, then put in the specifications for 5mL small beaker;(2) adding 0.65mL cold 0.9% saline in the beaker, and with ophthalmic scissors cutting brain block. as soon as possible; The brain tissue suspension was then poured into the homogenate tube, and the cold 0.86% saline 0.3mL was added to the homogenate of 3~5min, and the 10% brain tissue homogenate was prepared, and centrifuged at 12,000×g for 10 minutes at 4°C; The above steps were carried out on the ice.

Take proper amount of supernatant of SOD, MDA, GSH, GSH-Px detection, the specific methods of operation in strict accordance with the completion of Nanjing Institute of biological engineering kit (MDA:20140530; GSH:20140510; GSH-Px:20140512; SOD:20140508; Nanjing Jiancheng Biological Engineering Research Institute Co., Ltd. Nanjing, China) detection steps.

TUNEL assay

Select appropriate brain slices from each group of rats from the in situ hybridization protection solution. TUNEL staining was performed as described previously according to the manufacturers' protocols with minor modifications. Briefly, TUNEL assay was performed in 20-μm-thick frozen sections using in situ cell death detection kit (Roche Molecular Systems, Inc; Switzerland Basel, Germany). All images were acquired using a confocal microscope (Leica TCS SP2, Solms, Germany). The nuclei were stained with DAPI (blue), and the apoptotic cells appeared green. Image Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA) was used for cell counting.

Statistical analysis

The experimental data statistics are expressed as mean $\pm$ standard error (Means $\pm$ SEM). Two groups of data were compared using T test, and multiple groups of data were analyzed by One-way ANOVA or Two-way ANOVA followed with Turkey's multiple comparison test post hoc. When $P < 0.05$, there was statistical difference.

Results

CDG ameliorated behavioral symptoms of 6-OHDA induced PD rats

Rotational behavior directly reflects the degeneration and death of dopaminergic neurons in PD rats [24]. With induction of APO, no rotational behavior was observed throughout the test in sham-operated rats, while the number of the rotations of PD rats significantly increased after surgery and gradually decreased week by week ($P < 0.001$). The number of rotations of the Madopar group decreased significantly compared with 6-OHDA-lesioned group ($P < 0.01$) at 6 weeks. With 4 weeks, and 6 weeks of treatment, the number of rotations of the CDG group significantly decreased compared with the model group ($P < 0.001$, Fig 1). There was no significant in the number of rotations between the Madopar group and the CDG group ($P > 0.05$). These results suggested that CDG reduced motor dysfunction in PD rats.

CDG attenuated nigrostriatal dopamine loss of PD rats

Loss of striatal DA and its metabolites is closely associated with the dyskinesia in PD. In this study, the striatal contents of neurotransmitter DA and the intermediate metabolites including DOPAC and HVA were determine with HPLC-ECD. 6-OHDA toxicity significantly reduced the DA, DOPAC, and HVA levels in the striatum of model rats. Compared with the model group, the striatal contents of DA, DOPAC and HVA all increased significantly in both the Madopar and CDG group ($P < 0.01$, Fig 2A-C). However, the levels of each neurotransmitter were similar between the two groups ($P > 0.05$). These results indicated that CDG treatment could increase the contents of neurotransmitters including DA, DOPAC, and HVA in the striatum of 6-OHDA induced PD rat.

To examine the DA neuronal injuries in the SNpc of PD, the protein expression of TH in striatum was determined. Compared with the sham-operated groups, the expression of TH protein in the striatum of PD rats was significantly decreased ($P < 0.01$). After treatment with Madopar and CDG, the expression of TH protein was significantly improved ($P < 0.05$, $P < 0.01$, Figure 2D-E). Moreover, the expression of TH protein in CDG group was higher than that in Madopar group. However, there was no significant in them ($P > 0.05$). These dates suggested that CDG increased TH protein expression in PD rats.

CDG attenuated 6-OHDA induced loss of nigrostriatal DA neurons in the PD rats

Immunohistochemistry staining was used to evaluate the injuries of nigrostriatal DA neurons (Fig 3). The results showed that the DA neurons of SNpc in the sham-operated group were brown, plump, large in number, and clear in outline and process. In PD model rats, the cell bodies of neurons atrophied, and their

outlines and processes were unclear. The number of DA neurons in the SNpc of PD rats significantly decreased compared with the sham group. The number of DA cells in Madopar group increased significantly compared with the model group ($P < 0.05$). After treatment with CDG, the number of DA cells increased significantly compared with the model group. In addition, the average optical density of the striatum of PD model decreased, CDG and Madopar group could improve the average optical density of the striatum of PD rats. However, there was no significant in them ($P > 0.05$). These results indicated that CDG treatment could attenuate the 6-OHDA induced DA neuronal injury.

CDG alleviated 6-OHDA induced oxidative stress in the Striatum of PD rats

Evidences have been developed for oxidative damage to key cellular components in the SNpc of PD, ultimately leading to cell death. GSH reacts with H_2O_2 to produce oxidized glutathione, thus scavenging oxygen free radicals. Thereby GSH turns to indicate the antioxidant capacities of organisms [25]. Compared with the Sham group, the SOD content, GSH and GSH-Px activity in the striatum of the Model group reduced significantly, while the MDA level significantly increased; Compared with the model group, the MDA level of rats in the CDG and Madopar group decreased significantly, while the SOD content, GSH and GSH-Px activity increased significantly (Fig 4). However, there was no significant in CDG and Madopar group ($P > 0.05$). The results showed that CDG alleviated oxidative stress in PD rats.

CDG reduced 6-OHDA induced cell apoptosis in the nigrostriatal pathway of PD rats

The cell apoptosis in the SNpc of each group was stained and observed. Use TUNEL staining method to label apoptotic cells, and at the same time co-standardize with TH expression in the SNpc of rats. The experimental results showed that, compared with the sham group, the number of TUNEL positive cells in the model group was significantly increased. After 6 weeks of treatment, compared with the model group, the number of TUNEL positive cells in the SNpc was significantly reduced in the CDG and Madopar group (Fig 5A-B). There was no significant between CDG and Madopar group ($P > 0.05$). The results showed that CDG could suppress the apoptosis of PD rats.

Western Blotting analysis showed that a marked depletion in the expression of Bcl-2/Bax in the striatum of 6-OHDA-lesioned group compared to the sham group (Fig 5C-D, $P < 0.001$). After 6 weeks of treatment, Madopar group increased the protein expression of Bcl-2/Bax compared with 6-OHDA-lesioned group ($P < 0.05$). CDG also showed improvement in the level of striatal protein expression of Bcl-2/Bax ($P < 0.01$). The result showed that CDG inhibited the apoptosis of dopaminergic neurons in PD rats.

CDG increased the expression of neurotrophic factors in the SNpc of PD rats

BDNF, GDNF and NGF expression in the SNpc were visualized by IHC staining and the positive cells were counted (Fig 6). NGF-positive neurons and BDNF mainly expressed in the cytoplasm. GDNF mainly exists in the nucleus of neuronal cells. Compared to the Sham group, the number of NGF, BDNF, and GDNF-positive cells in the SNpc of PD model rats significantly decreased ($P < 0.001$). After 6 weeks of intervention, the counts of NGF, BDNF and GDNF in the SNpc of the Madopar group and CDG group were

all higher than those in PD model group. There was no significant difference between Madopar group and CDG group ($P > 0.05$). The results showed that CDG increased the expression of neurotrophic factors (NGF, BDNF, GDNF) in the SNpc of PD rats.

Discussion

The incidence of PD increases greatly in the worldwide. Levodopa preparations and anticholinergic drugs have been commonly used in PD therapy as far. However, the side effects are usually reported within 2-5 years of medicine treatment and increases along with the course. An approved alternative drug specifically targeted to PD is almost nonexistent at present [26]. A large number of studies have shown that TCM treatment could not only improve the clinical efficacy but also reduce the side effects of chemically synthesized drugs in PD therapy [27]. CDG had a good therapeutic efficacy on PD [28,29]. In our previous work, CDG reduced the adverse effects of L-DOPA in PD rat model by increasing the expression of DAT [30]. In this study, we obtained experimental evidence that CDG improved motor function of PD rats, as well as the increased DA neurons, consistently demonstrating the protective role of CDG in PD.

Unilateral injection of 6-OHDA was performed to set up a rat model of PD, which is the most common and widely used model to explore the efficacy of PD drugs. The protective effects and molecular mechanisms of the sulfated polysaccharide (SJP) against 6-OHDA-induced toxicity were investigated in SH-SY5Y cells [21]. Wang used 6-OHDA induced PD rats to analyze the effect of electroacupuncture on alleviating motor symptoms and up-regulating vesicular glutamatergic transporter 1 expression [32]. In our study, the 6-OHDA induced PD rats were used, in which the rotational behaviors were alleviated, and the number of TH positive neurons, the expression of TH in the striatum and the contents of neurotransmitters including DA, DOPAC, and HVA were increased significantly with CDG treatment.

Madopar, containing two active ingredients called levodopa and benserazide, is one of the antiparkinsonian agents frequently used in PD to increase the levels of dopamine in the brain. Madopar was commonly used as a control group by improving motor symptoms and increasing the number of DA neurons in rats with PD [33-35]. Madopar was also applied in this study as a positive control for PD treatment, the 6-week treatment of which delivered similar improvements in 6-OHDA induced PD rats.

Although the specific pathogenesis and progression of PD are not fully understood, overwhelming evidences suggest that oxidative stress plays an important role in dopaminergic neuronal degeneration [8]. The maintenance of redox potential is an important factor for neuronal survival, any disruption in which might interfere with other cellular biological processes, ultimately leading to cell death [36]. Satpute' research investigated that resolved the oxidative stress could protect tyrosine hydroxylase-positive neurons in midbrain [34]. In this study, the enhanced level of MDA, decreased SOD content and GSH and GSH-Px activity in the striatum of 6-OHDA induced PD rats were all reversed by CDG treatments.

The Bcl-2 protein family plays a major role in the process of apoptosis. It contains both pro-apoptotic proteins (such as Bax) and anti-apoptotic proteins (such as Bcl-2) [37]. When stimulated by various

factors (such as oxidative stress), pro-apoptotic proteins will translocate to the outer membrane of mitochondria, triggering the release of cytochrome C and other apoptosis-inducing factors, inducing apoptosis; In contrast, the inhibitor of apoptosis protein factor will inhibit the occurrence of apoptosis [38]. And we found that CDG was able to reduce the number of TUNEL-positive neurons in the SNpc, and increase the expression of Bcl-2/Bax protein. Neurotrophic factor (NTF) is a peptide active substance that can stimulate the regeneration of adult neurons, play a neuroprotective role, and reduce the death of DA neurons. It has been reported that NGF has a protective effect on PC12 cell death caused by 6-OHDA and reduces the occurrence of apoptosis [39]. BDNF can promote the survival, differentiation and growth of DA neurons [40]. Experimental studies showed that GDNF was injected into the SNpc of PD model rat injured by 6-OHDA, and its behavior and DA content were improved [39]. In this experiment, we observed increased oxidative stress and apoptosis in PD rats, and CDG could ease oxidative stress, alleviate apoptosis, increase the expression of NGF, BDNF and GDNF in the SNpc of PD rats. thereby exerting a neuroprotective effect, but its specific mechanism still needs in-depth research.

With TCM theory, some evidences confirmed that a variety of Chinese herbs and their effective components have the effect of delaying the progression of PD. Wang found that CDG could improve the oxidative stress of PD rats, thus playing the role of "enhancing the effect and reducing the toxicity" [41]. Hu found that CDG regulated the expression of apoptosis-related proteins through the PI3K/Akt signaling pathway and inhibited the apoptosis of the striatum cells of PD rats [42]. Liang's research investigated that CDG played a role in the treatment of PD by regulating the expression of neurotrophic factor and its receptor [43]. Zheng's study confirmed that paeoniflorin could ameliorate the behavioral deficits and reduce DA cell loss that were induced by MPTP in the model of PD [44]. Li's study found that isorhynchophylline significantly attenuated 1-methyl-4-phenylpyridinium (MPP⁺)-induced apoptotic cell death and oxidative stress in PC12 cells [45]. The neuroprotective effects of verbascoside on the neuronal apoptosis and oxidative stress of MPP⁺-induced PC12 were verified [46]. This suggested that these compounds may be the material basis of the activity of CDG against PD.

In this study, we demonstrated that CDG could improve the neurobehavioral performance of PD rats and increase the expression of TH protein, TH⁺ neurons and the DA, DOPAC and HVA of PD rats. Moreover, the oxidative stress and the cell apoptosis induced by 6-OHDA toxicity were also ameliorated by CDG treatment. CDG increased the expression of NGF, BDNF and GDNF in the SNpc of PD rats. Collectively, CDG is of potential therapeutic value in the treatment of PD. In the future, we would split and recombine the herbs in CDG, to explore the further mechanism and to establish the potential active components.

Conclusions

CDG treatment can alleviate DA neuron loss and motor deficits of PD by ameliorating the oxidative stress, inhibiting the cell apoptosis and increasing the expression of neurotrophic factors.

Abbreviations

PD: Parkinson's disease; CDG: Compound Dihuang Granule; DA: Dopamine; SNpc: Substantia nigra pars compacta; DOPAC: 3,4-dihydroxyphenylacetic acid; HVA: Homovanillic acid; IHC: Immunohistochemistry; DRT: Dopamine replacement therapy; TCM: Traditional Chinese medicine; HPLC: High performance liquid Chromatography; EC: Electrochemical detection; RT: Room temperature; BSA: Bovine serum albumin; SOD: Supernatant of superoxide dismutase; MDA: Malondialdehyde; GSH: Glutathione; GSH-Px: Glutathione peroxidase; APO: Apomorphine; DAT: Dopamine transporter; 6-OHDA: 6-hydroxydopamine; TH: Tyrosine hydroxylase; Bax: Bcl-2 associated X; Bcl-2: B-cell lymphoma-2; DAB: 3,3'-Diaminobenzidine; DAPI: 4,6-Diamidino-2-Phenylindole; TUNEL: Transferase-mediated deoxyuridine triphosphate-biotin nick end labeling; MPTP: 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine;

Declarations

Acknowledgments

We are very grateful to Professor Chan-xing Yuan of Shanghai longhua Hospital, China, for providing suggestions in the experimental design.

Author contributions

Each author has contributed significantly to this study. YFY and JCH conceived and designed the study. LW performed the animal and molecular biology experiments. DYB, LZ, ZQH performed the statistical analyses. LW drafted the manuscript. LW, YFY and JCH revised the manuscript. All authors read and approved the final manuscript.

Funding

This work was funded by the National Natural Science Foundation of China (No.81573899), the Shanghai Sailing Program (No.19YF1448500) and the General project of China Postdoctoral Science Foundation (No.2019M651562). The funding sources had no role in study conception and design, data analysis or interpretation, paper writing or deciding to submit this paper for publication.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All experimental procedures were conducted according to the National Institute of Health Guide for the Care and Use of Laboratory Animals, and approved by the Animal Care Committee of Shanghai University of TCM.

Consent for publication

All authors agree to publish this paper.

Competing interests

The authors declare that they have no competing interests.

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References

1. Przedborski, S (2017) The two-century journey of parkinson disease research. *Nat.Rev. Neurosci.* 18,251-259.
2. Kalia L V, Lang A E (2015) Parkinson's disease. *Lancet* 386: 896-912.
3. Lohle M, Reichmann H. Clinical neuroprotection in Parkinson's disease - still waiting for the breakthrough. *J Neurol Sci* 2010;289(1-2):104-14.
4. Cao XB, Guan Q, Xu Y, Wang L, Sun SG. Mechanism of over-activation in direct pathway mediated by dopamine D(1) receptor in rats with levodopa-induced dyskinesia. *Neurosci Bull* 2006;22(3):159-64.
5. Fahn S, Oakes D, Shoulson I, Kieburtz K, Rudolph A, Lang A, Olanow CW, Tanner C, Marek K. Levodopa and the progression of Parkinson's disease. *N Engl J Med* 2004;351(24):2498-508.
6. Pahwa R, Factor SA, Lyons KE, Ondo WG, Gronseth G, Bronte-Stewart H, Hallett M, Miyasaki J, Stevens J, Weiner WJ. Practice Parameter: treatment of Parkinson disease with motor fluctuations and dyskinesia (an evidence-based review): report of the Quality Standards Subcommittee of the American Academy of Neurology. *Neurology* 2006;66(7):983-95.
7. Hobson DE, Lang AE, Martin WR, Razmy A, Rivest J, Fleming J. Excessive daytime sleepiness and sudden-onset sleep in Parkinson disease: a survey by the Canadian Movement Disorders Group. *JAMA* 2002;287(4):455-63.
8. Trist B G, Hare D J, Double K L. Oxidative stress in the aging substantia nigra and the etiology of Parkinson's disease[J]. *Aging Cell*, 2019, 18(6): e13031.
9. Lin C H, Wei P C, Chen C M, et al. Lactulose and Melibiose Attenuate MPTP-Induced Parkinson's Disease in Mice by Inhibition of Oxidative Stress, Reduction of Neuroinflammation and Up-Regulation of Autophagy[J]. *Front Aging Neurosci*, 2020, 12: 226.
10. Erekat N S: Apoptosis and its Role in Parkinson's Disease, Stoker T B, Greenland J C, editor, *Parkinson's Disease: Pathogenesis and Clinical Aspects*, Brisbane (AU), 2018.
11. Zhou F, Ju J, Fang Y, et al. Salidroside protected against MPP(+)-induced Parkinson's disease in PC12 cells by inhibiting inflammation, oxidative stress and cell apoptosis[J]. *Biotechnol Appl Biochem*, 2019, 66(2): 247-253.

12. Ge B, Li S L, Li F R. Astragaloside-IV regulates endoplasmic reticulum stress-mediated neuronal apoptosis in a murine model of Parkinson's disease via the lincRNA-p21/CHOP pathway[J]. *Exp Mol Pathol*, 2020, 115: 104478.
13. Anusha C, Sumathi T, Joseph L D. Protective role of apigenin on rotenone induced rat model of Parkinson's disease: Suppression of neuroinflammation and oxidative stress mediated apoptosis[J]. *Chem Biol Interact*, 2017, 269: 67-79.
14. Chmielarz P, Saarma M. Neurotrophic factors for disease-modifying treatments of Parkinson's disease: gaps between basic science and clinical studies[J]. *Pharmacol Rep*, 2020.
15. Shadfar S, Kim Y G, Katila N, et al. Neuroprotective Effects of Antidepressants via Upregulation of Neurotrophic Factors in the MPTP Model of Parkinson's Disease[J]. *Mol Neurobiol*, 2018, 55(1): 554-566.
16. Antunes M S, Cattelan Souza L, Ladd F V L, et al. Hesperidin Ameliorates Anxiety-Depressive-Like Behavior in 6-OHDA Model of Parkinson's Disease by Regulating Striatal Cytokine and Neurotrophic Factors Levels and Dopaminergic Innervation Loss in the Striatum of Mice[J]. *Mol Neurobiol*, 2020, 57(7): 3027-3041.
17. Liang JQ, He JC. Effect of compound Dihuang Granule on neurobehavior of parkinsonian dyskinesia model rats with Yin deficiency and moving wind syndrome [J]. *Chinese PLA medical Journal*, 2018,43 (08): 652-656.
18. Wang L Y, Yu X, Li X X, et al. Catalpol Exerts a Neuroprotective Effect in the MPTP Mouse Model of Parkinson's Disease[J]. *Front Aging Neurosci*, 2019, 11: 316.
19. Liang JQ, Wang L, He JC, Hua XD. Verbascoside promotes the regeneration of tyrosine hydroxylase-immunoreactive neurons in the substantia nigra. *Neural Regen Res* 2016;11(1):101-06.
20. Tseng YT, Hsu YY, Shih YT, Lo YC. Paeonol attenuates microglia-mediated inflammation and oxidative stress-induced neurotoxicity in rat primary microglia and cortical neurons. *Shock* 2012;37(3):312-18.
21. Jiang J, Kim J J, Kim D Y, et al. Acorus gramineus inhibits microglia mediated neuroinflammation and prevents neurotoxicity in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced mouse model of Parkinson's disease[J]. *J Ethnopharmacol*, 2012, 144(3): 506-13.
22. Jing X, Wei X, Ren M, et al. Neuroprotective Effects of Tanshinone I Against 6-OHDA-Induced Oxidative Stress in Cellular and Mouse Model of Parkinson's Disease Through Upregulating Nrf2[J]. *Neurochem Res*, 2016, 41(4): 779-86.
23. Doyle SE, Grace MS, McIlvor W, Menaker M. Circadian rhythms of dopamine in mouse retina: the role of melatonin. *Vis Neurosci* 2002;19(5):593-601.
24. Grealish S, Mattsson B, Draxler P, Bjorklund A. Characterisation of behavioural and
25. neurodegenerative changes induced by intranigral 6-hydroxydopamine lesions in a mouse model of Parkinson's disease. *Eur J Neurosci* 2010;31(12):2266-78.
26. Trevisan M, Browne R, Ram M, Muti P, Freudenheim J, Carosella AM, Armstrong D. Correlates of markers of oxidative status in the general population. *Am J Epidemiol* 2001;154(4):348-56.

27. Ho AL, Ali R, Connolly ID, Henderson JM, Dhall R, Stein SC, Halpern CH. Awake versus asleep deep brain stimulation for Parkinson's disease: a critical comparison and meta-analysis. *J Neurol Neurosurg Psychiatry* 2018;89(7):687-91.
28. Zhao G. Further discussion on the three methods for the stage-treatment of Parkinson's disease. *J Tradit Chin Med* 2008;28(1):39-43.
29. Chen S, Le W. Neuroprotective therapy in Parkinson disease. *Am J Ther* 2006;13(5):445-57.
30. He JC. Re-thinking of treatment of Parkinson's disease in traditional Chinese medicine—Discussion on three basic therapeutic rules of the disease, reinforcing kidney & smoothing liver, activating blood & dissolving stasis and dispelling phlegm & clearing nodules. *Zhongyiyao Tongbao* 2005;(1):12-14.
31. Luo, RJ; He, JC .Effects of compound rehmannia formula on dopamine transporter content in the corpus striatum of Parkinson's disease rats treated with levodopa , *Neural Regeneration Research* 2011;6(12),898-902.
32. Cui C, Cui N, Wang P, Song S, Liang H, Ji A. Neuroprotective effect of sulfated polysaccharide isolated from sea cucumber *Stichopus japonicus* on 6-OHDA-induced death in SH-SY5Y through inhibition of MAPK and NF-kappaB and activation of PI3K/Akt signaling pathways. *Biochem Biophys Res Commun* 2016;470(2):375-83.
33. Wang Y, Wang Y, Liu J, Wang X. Electroacupuncture Alleviates Motor Symptoms and Up-Regulates Vesicular Glutamatergic Transporter 1 Expression in the Subthalamic Nucleus in a Unilateral 6-Hydroxydopamine-Lesioned Hemi-Parkinsonian Rat Model. *Neurosci Bull* 2018;34(3):476-84.
34. Wang JY, Yang JY, Wang F, Fu SY, Hou Y, Jiang B, Ma J, Song C, Wu CF. Neuroprotective effect of pseudoginsenoside-f11 on a rat model of Parkinson's disease induced by 6-hydroxydopamine. *Evid Based Complement Alternat Med* 2013;2013:152798.
35. Satpute R, Lomash V, Kaushal M, Bhattacharya R. Neuroprotective effects of alpha-ketoglutarate and ethyl pyruvate against motor dysfunction and oxidative changes caused by repeated 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine exposure in mice. *Hum Exp Toxicol* 2013;32(7):747-58.
36. Ren R, Shi C, Cao J, Sun Y, Zhao X, Guo Y, Wang C, Lei H, Jiang H, Ablat N, Xu J, Li W, Ma Y, Qi X, Ye M, Pu X, Han H. Neuroprotective Effects of A Standardized Flavonoid Extract of Safflower Against Neurotoxin-Induced Cellular and Animal Models of Parkinson's Disease. *Sci Rep* 2016;6:22135.
37. Baluchnejadmojarad T, Mansouri M, Ghalami J, Mokhtari Z, Roghani M. Sesamin imparts neuroprotection against intrastriatal 6-hydroxydopamine toxicity by inhibition of astroglial activation, apoptosis, and oxidative stress. *Biomed Pharmacother* 2017;88:754-61.
38. Banjara S, Suraweera CD, Hinds MG, Kvensakul M. The Bcl-2 Family: Ancient Origins, Conserved Structures, and Divergent Mechanisms. *Biomolecules* 2020;10(1).
39. Finlay D, Teriete P, Vamos M, Cosford N, Vuori K. Inducing death in tumor cells: roles of the inhibitor of apoptosis proteins. *F1000Res* 2017;6:587.
40. Yamada K, Umegaki H, Maezawa I, Iguchi A, Kameyama T, Nabeshima T. Possible involvement of catalase in the protective effect of interleukin-6 against 6-hydroxydopamine toxicity in PC12 cells. *Brain Res Bull* 1997;43(6):573-77.

41. Hagg T. Neurotrophins prevent death and differentially affect tyrosine hydroxylase of adult rat nigrostriatal neurons in vivo. *Exp Neurol* 1998;149(1):183-92.
42. Wang L, He, JC. Intervention of Compound Dihuang Granules on Oxidative Stress in Parkinson 's Disease Rats. *Shi Zhen Guo Yao Guo Yao* 2019;30(12): 2840-2842.
43. Hu D, Teng L, Hong F, He, JC. Effect of Fufang Dihuang Granules on Apoptosis of Striatum Cells and PI3K / Akt Signaling Pathway in Parkinson 's Disease Rats with Yin Deficiency and Wind Syndrome, *Clinical and Experimental Medical Journal* 2019;18 (03),240-245.
44. Liang JQ, He, JC. Effects of Fufang Dihuang Granules on neurotrophic factors and their receptors in rats with Parkinson's disease model of Yin deficiency and dynamic wind syndrome, *PLA Journal of Medical Sciences* 2018;43 (08),647-651.
45. Zheng M, Liu C, Fan Y, Yan P, Shi D, Zhang Y. Neuroprotection by Paeoniflorin in the MPTP mouse model of Parkinson's disease. *Neuropharmacology* 2017;116:412-20.
46. Li XM, Zhang XJ, Dong MX. Isorhynchophylline Attenuates MPP(+)-Induced Apoptosis Through Endoplasmic Reticulum Stress- and Mitochondria-Dependent Pathways in PC12 Cells: Involvement of Antioxidant Activity. *Neuromolecular Med* 2017;19(4):480-92.
47. Sheng GQ, Zhang JR, Pu XP, Ma J, Li CL. Protective effect of verbascoside on 1-methyl-4-phenylpyridinium ion-induced neurotoxicity in PC12 cells. *Eur J Pharmacol* 2002;451(2):119-24.

Tables

Table 1 Pharmaceutical ingredients of Compound Dihuang Granule

Latin name	Species	Family	Part used
Rehmanniae radix praeparata	Rehmannia glutinosa Libosch.	Scrophulariaceae	Rootstock
Paeoniae radix alba	Paeonia lactiflora Pall.	Ranunculaceae	Roots
Uncariae ramulus cum uncis	Unacaria rhynchophylla (Miq.) Miq.ex Havil.	Rubiaceae	Stem and branch
Margaritifera concha	Hyriopsis cumingii (Lea)	Mussels	Shell
Salviae miltiorrhizae radix et rhizoma	Salvia miltiorrhiza Bge.	Labiatae	Roots
Acori tatarinowii rhizoma	Acorus tatarinowii Schott	Araceae	Rootstock
Scorpio	Buthus martensii Karsch	Buthidae	Dry body

The ratio of these herbs was 20:30:15:15:20:12:2.

Figures

Figure 1

Behavioral performances of rats in the rotation test. Rotating laps of rats with the induction of apomorphine were examined in 0 week, 2 weeks, 4 weeks and 6 weeks after CDG treatments. Statistical analysis was performed with Two-Way ANOVA, $n=9$. Significant differences were indicated by ** $P \leq 0.01$; *** $P \leq 0.001$.

Figure 2

CDG against 6-OHDA induced dopamine reduction and TH-positive neuron loss. (A-C) Contents of striatum DA (A), DOPAC (B) and HVA (C) were analyzed by HPLC-ECD; (D, E) TH protein expression was examined by western blotting (D) and TH protein was quantified (E). GAPDH served as control. Statistical analyses were performed with One-Way ANOVA, Turkey's multiple comparison test post hoc, $n = 3$. Significant differences were indicated by * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

Figure 3

6-OHDA induced loss of dopaminergic neurons in the nigrostriatal pathway of the rat brain. (A) DAB staining of TH on midbrain sections in each group (Scale bar: 400µm). (B) DAB staining of enlarged TH-positive cells in each group (Scale bar: 100µm). (C) Stereological counts of TH-positive cells of the SNpc at 6 weeks after 6-OHDA intoxication. (D) Average optical density of striatum in each group. Statistics of

the number of TH positive neurons was shown beside. Statistical analysis was performed with Two-Way ANOVA, n = 3. Significant differences were indicated by * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Figure 4

The relative activity of molecules involved in oxidative stress. The SOD content, the activities of GSH and GSH-Px , the SOD content and the MDA level of the striatum were detected. Statistical analyses were performed with One-Way ANOVA, Turkey’s multiple comparison test post hoc, n=9. Significant differences were indicated by * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Figure 5

(A) TUNEL assay of CDG reduced apoptosis in 6-OHDA induced PD rats. representative confocal fluorescent images of the SNpc with TUNEL (green), TH (red) and DAPI (blue). Scale bar: 100 μ m. (B) The apoptosis index of the SNpc at 6 weeks after 6-OHDA intoxication. (C) The expression levels of Bax, Bcl-2 proteins of the striatum. The protein expression was examined by Western blotting. GAPDH served as control. (D) The expression of Bcl-2/Bax in the striatum. Statistical analysis was performed with One-Way

ANOVA, Turkey's multiple comparison test post hoc, or Two-Way ANOVA, n =3. Significant difference was indicated by * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

Figure 6

Immunohistochemical staining labeled neurotrophic factors in SNpc in 6-OHDA induced PD rats. (A) IHC staining of NGF, BDNF and GDNF in the SNpc of each group. NGF, BDNF and GDNF were indicated with IHC in brown; the nucleus of cells were counterstained with hematoxylin (deep purple). Scale bar: 100µm. (B-D) Stereological counts of NGF, BDNF and GDNF positive cells of the SNpc at 6 weeks after 6-OHDA intoxication. Statistical analysis was performed with Two-Way ANOVA, n = 3. Significant differences were indicated by * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

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