Neuroprotective Effect of Gypenosides against Oxidative Injury in the Substantia Nigra of a Mouse Model of Parkinson’s Disease

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Oxidative injury has been implicated in the aetiology of Parkinson’s disease (PD) and gypenosides (GP), which are saponins with various bioactivities, have shown antioxidative effects in vitro. The present study was designed to evaluate the effect of GP on a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced mouse model of PD. Acute administration of MPTP led to decreased glutathione content and reduced superoxide dismutase activity in the substantia nigra of the mice, which resulted in oxidative stress, loss of nigral dopaminergic neurons and motor dysfunction. Co-treatment with GP attenuated all the injuries induced by MPTP in a dose-dependent manner. The neuroprotective effect of GP may be attributed to increased antioxidation, as manifested by significantly increased glutathione content and enhanced superoxide dismutase activity in the substantia nigra. These results strongly indicate the possible therapeutic potential of GP as an antioxidant in PD.

KEY WORDS: GYPENOSIDES; PARKINSON’S DISEASE; OXIDATIVE STRESS; DOPAMINE; GLUTATHIONE; SUPEROXIDE DISMUTASE

Introduction

Parkinson’s disease (PD) is a common neurodegenerative disease characterized by progressive movement disorder and loss of dopaminergic neurons in the substantia nigra pars compacta (SNc). A growing body of evidence indicates that oxidative stress may be an important contributor to dopaminergic neuronal death during PD. The occurrence of oxidative stress in PD has been supported by studies on nigral tissues showing reduced levels of endogenous antioxidant molecules such as glutathione (GSH), decreased activity of antioxidant enzymes such as superoxide dismutase (SOD), increased lipid peroxidation and elevated levels of oxidative injury products such as 4-hydroxynonenal (HNE), protein carbonyls and 8-hydroxyguanine (8-OHG).

Gypenosides (GP), which are saponins extracted from Gynostemma pentaphyllum (the twisting vine orchid, a plant indigenous to the southern reaches of China, South Korea and Japan), have shown various...
bioactivities such as hepatoprotection,\textsuperscript{12} antihyperlipidaemia\textsuperscript{13,14} and anticancer effects.\textsuperscript{15} Although GP has a therapeutic benefit for some diseases, to the best of the authors’ knowledge the effect of GP on PD is still unknown. It has been shown, \textit{in vitro}, that GP can protect rat cortical cells against glutamate-induced oxidative injury by preventing GSH depletion and lipid peroxidation.\textsuperscript{16}

The present study was designed to investigate whether GP had any neuroprotective effect for PD \textit{in vivo} by preventing oxidative stress in the SNc. A 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced mouse model\textsuperscript{1} of PD was used to assess behaviour, neurochemistry and oxidative stress markers.

**Materials and methods**

**ANIMALS AND CHEMICALS**

Male C57BL/6 10-week old mice, weighing 20 – 25 g were used. All procedures were approved by the Committee for Animal Use for Research and Education at the Fourth Military Medical University, Xi’an, China, and all efforts were made to minimize the number of animals used and their suffering. GP (purity > 99\%), confirmed by high-performance liquid chromatography [HPLC] analysis; Ankang Pharmaceutical Co., Shanxi, China) and MPTP (Sigma-Aldrich, St Louis, MO, USA) were dissolved in sterile saline at concentrations of 20 and 10 mg/ml, respectively.

**EXPERIMENTAL DESIGN**

The mice were randomly assigned to five groups (n = 12 mice/group): group 1, control (sham); group 2, PD + saline; group 3, PD + GP 100 mg/kg; group 4, PD + GP 200 mg/kg; and group 5, PD + GP 400 mg/kg. Mice in groups 2 – 5 received four intraperitoneal doses of 20 mg/kg of MPTP at 2-h intervals to induce PD. At 1 h after the final injection, these mice received different doses of GP intraperitoneally, according to the schedule above. Control mice received an equivalent volume of sterile saline solution intraperitoneally at each injection point. After 3 days, the mice were given a behaviour test (n = 12 mice/group). Six of the 12 mice from each group were then perfused transcardially with paraformaldehyde and the midbrain was removed and cut coronally at 20 µm. All sections were collected in 0.01 M phosphate-buffered saline (pH 7.4). The other six mice from each group were sacrificed to harvest fresh brain tissue for other assays.

**BEHAVIOURAL TESTS**

**Pole test\textsuperscript{17}**

Mice were placed head upwards on top of a vertical wooden pole 50 cm long and 1 cm in diameter. The time taken for the mouse to turn nose down (inversion time) and the total time required for the mouse to climb down the pole were measured. Five trials were recorded and the mean value calculated for each mouse (n = 12 mice/group).

**Rotarod test\textsuperscript{18}**

For this test, mice were positioned on a Rotarod (IITC Life Science Inc., Woodland Hills, CA, USA), which was rotated with increasing speed. The total distance moved before the mice fell off the rod was automatically calculated. The mean of five trials was calculated for each mouse (n = 12 mice/group).

**TH IMMUNOHISTOCHEMISTRY AND TUNEL STAINING**

**TH immunolabelling**

Briefly, midbrain sections of 20 µm from six mice from each group were treated with a blocking serum (30% goat serum) for 1 h at room temperature. After washing in 0.01 M phosphate-buffered saline (PBS), pH 7.4, the
sections were incubated at room temperature for 24 h with mouse antiserum against tyrosine hydroxylase (TH) (1:1000 dilution; Sigma-Aldrich®), then washed for three times in 0.01 M PBS before incubation with a secondary Cy3-labelled donkey antimouse immunoglobulin (Ig) G (1:200 dilution; Millipore, Billerica, MA, USA) for another 4 h at room temperature. Subsequently, sections were washed three times in 0.01 M PBS, air-dried, cover-slipped with a mixture of 50% (v/v) glycerin and 2.5% (w/v) triethylenediamine (antifading agent) in 0.01 M PBS, and observed with a confocal laser scanning microscope (Olympus FV1000; Tokyo, Japan). The number of positive neurons (red fluorescence) was counted.

**TUNEL staining**

The mouse midbrain sections from six mice from each group were stained using a terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick-end labelling (TUNEL) kit (Roche Diagnostics, Mannheim, Germany) as described previously.19 After staining, all sections were observed with the confocal laser scanning microscope and the number of apoptotic cells (green fluorescence) were counted.

**DOPAMINE ASSAY**

The brains of six mice from each group were rapidly removed and the bilateral striatum dissected and homogenized. The supernatant was used for HPLC to estimate dopamine levels, expressed as ng/g wet weight of tissue, as described previously.20 Samples were quantified using EuroChrom® 2000 software, version 10.0 (Knauer, Berlin, Germany) for Windows®.

**OXIDATIVE STRESS ESTIMATION**

The bilateral SNc was dissected from the brains of six mice from each group using the procedure described by Smith and Cass.21 In brief, the animals were rendered unconscious with carbon dioxide, decapitated, and the brains quickly removed and chilled in ice-cold saline. A coronal slice was made through the midbrain and the bilateral SNc were removed from both sides of the brain. They were then homogenized in cold 0.32 M sucrose buffer containing protease inhibitors using a sonic dismembrator and centrifuged for 15 min at 1000 g. The supernatant was transferred to a clean vial for oxidative stress analysis.

**GSH determination**

The GSH content was measured as described previously22 by using 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB). This reduces GSH to form the chromophoric product, 2-nitro-5-thiobenzoic acid, which can then be detected spectrophotometrically at 412 nm. The GSH content in the bilateral SNc samples in the present study was determined using a standard curve for GSH and was expressed in nmol/mg protein.

**SOD activity assay**

The activity of SOD in the bilateral SNc samples was measured using a previously described assay;23 the photo-oxidation of hydroxylamine hydrochloride generates superoxide anions that can be measured quantitatively as they reduce nitroblue tetrazolium dye, and SOD acts as an inhibitor of this reaction. The results in the present study were expressed as U/mg protein.

**Assays for protein carbonyls and HNE**

Whole tissue homogenate (20 mg) of bilateral SNc was applied to a nitrocellulose membrane via vacuum filtration for 0.5 h. First, the membranes were blocked with 30% goat serum for 1 h at room temperature. After washing in 0.01 M PBS, pH 7.4, the
membranes were incubated at room temperature with rabbit antidinitrophenol (DNP; 1:150 dilution; Millipore) or rabbit antiHNE (1:1000 dilution; Millipore) for 24 h, then washed three times in 0.01 M PBS before incubation with a secondary goat antirabbit IgG conjugated to alkaline phosphatase (1:200 dilution; Sigma-Aldrich®) for another 4 h at room temperature. Finally, the membranes were washed three times in 0.01 M PBS and then reacted with an alkaline phosphatase substrate (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium chloride liquid substrate system; Sigma-Aldrich) to visualize the resulting protein bands. After drying, the bands were quantified by densitometry as described previously.24

8-OHG assay
Oxidative DNA damage was measured using an 8-OHG assay, as described previously.11 Gas chromatography–mass spectrometry was used to examine the concentration of 8-OHG, which was expressed as nmol/mg DNA.

Lipid peroxidation assay
Polyunsaturated fatty acids are highly susceptible to peroxidation. Cycloperoxides are formed as a result of the peroxidation reaction, which gives malondialdehyde (MDA) by cleavage. The MDA forms a pink-coloured complex with thiobarbituric acid, the absorbance of which can be read at 532 nm. The lipid peroxidation level per sample of mouse tissue supernatant was measured as described previously25 and expressed as nmol of MDA formed/mg protein.

STATISTICAL ANALYSIS
The results are presented as mean ± SE. All analyses were carried out using the SPSS® statistical package, version 12.0 (SPSS Inc., Chicago, IL, USA) for Windows®. Statistical analysis of the data was carried out with a one-way analysis of variance followed by a Bonferroni post-hoc analysis. A P-value < 0.05 was considered to be statistically significant.

Results
MPTP-INDUCED MOTOR DEFICITS AND STRIATAL DOPAMINE LOSS
The pole test results showed a significant increase in the total time and the inversion time for the PD + saline group mice compared with the control group mice (P < 0.05). The distance moved in the Rotarod test and the striatal dopamine level were both significantly decreased in the PD + saline group compared with the control group mice (P < 0.05). In the groups treated with GP, the MPTP-induced motor deficits and striatal dopamine loss were significantly attenuated in a dose-dependent manner (P < 0.05; Fig. 1).

MPTP-INDUCED LOSS OF TH-POSITIVE NEURONS AND APOPTOSIS IN THE SNC
The number of TH-positive neurons was significantly decreased in the PD + saline group compared with the control group (P < 0.05), whereas GP treatment significantly increased the number of TH-positive neurons in a dose-dependent manner (P < 0.05) (Fig. 2A). Apoptosis levels increased significantly in the PD + saline group versus the control group (P < 0.05), whereas treatment with GP significantly decreased the number of apoptotic cells in a dose-dependent manner (P < 0.05) (Fig. 2B).

MPTP-INDUCED OXIDATIVE INJURY
Significantly decreased glutathione content (Fig. 3A) and SOD activity (Fig. 3B), significantly increased oxidative injury products such as protein carbonyls (Fig. 3C), HNE (Fig. 3D) and 8-OHG (Fig. 3E), and
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significantly increased lipid peroxidation products such as MDA (Fig. 3F) were detected in the bilateral SNc in the PD + saline group compared with the control group ($P < 0.05$ for all comparisons). Systemic administration of GP protected the SNc against oxidative injury in a dose-dependent manner ($P < 0.05$) (Fig. 3A–3F).

Discussion

Parkinson’s disease, a neurodegenerative disorder, is characterized by the progressive death of dopaminergic neurons in the SNc and a severe decrease in striatal dopamine. Although dopamine replacement therapy with l-3,4-dihydroxyphenylalanine (l-DOPA) is initially effective, the efficacy of l-DOPA gradually decreases and is often associated with severe side-effects. Intracerebral transplantation of fetal mesencephalic cells has been used to elevate striatal dopamine levels, but poor clinical
outcomes and ethical issues limit this approach. Studies have demonstrated that oxidative stress appears to be the aetiological factor in dopaminergic neuronal death during PD because post-mortem nigral tissue from PD patients has been shown to contain decreased GSH and SOD, and increased oxidative stress products. Thus, it has become an important objective to use an antioxidant in the aetiological treatment for PD.

Gypenosides, traditional Chinese drugs with various bioactivities, are liposoluble and pass easily through the blood–brain barrier. A previous in vitro study showed that GP could offer neuroprotection for cultured neurons through their antioxidant properties. In the present study, it was...
found that intraperitoneal administration of GP could significantly attenuate MPTP-induced motor deficits and striatal dopamine loss in a PD mouse model. The MPTP-induced loss of TH-positive neurons and apoptosis in SNc were also reversed by treatment with GP. Most importantly, GP treatment protected the SNc against MPTP-induced oxidative injury in a dose-dependent manner. Thus, the present study...
provides the first evidence that GP has neuroprotective effects against oxidative injury in the SNc in a mouse model of PD.

Superoxide dismutase is a primary cellular defence enzyme involved in protecting cells from oxidative stress in the brain. GSH, serving as a hydrogen donor, also plays a very important role in the protection of cells against the deleterious effects of free radicals. GSH is synthesized de novo from its three constituent amino acids by γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthetase (GS). Besides de novo synthesis, GSH is also generated from glutathione disulphide, catalysed by glutathione disulphide reductase (GR).

Normally, any superoxide anion generated in the brain is first converted into hydrogen peroxide by SOD, then GSH donates hydrogen to convert hydrogen peroxide into water as the final product. In the present study, the intraperitoneal injections of MPTP significantly decreased SOD activity and GSH content in the SNc, leading to oxidative stress, as shown by elevated levels of oxidative injury products such as protein carbonyls, HNE and 8-OHG. The GP treatment resulted in significantly higher SOD activity and a remarkable increase in GSH in the SNc compared with the mice not treated with GP. The enhanced antioxidative effects in the GP-treated mice contributed to the attenuated oxidative injury results, as shown by decreased oxidative injury products, reduced neuronal cell death, and reduced motor dysfunction compared with saline-treated mice.

Due to the good liposolubility of GP, it may diffuse directly into cellular nuclei to trigger the expression of γ-GCS, GS, GR or SOD genes. Alternatively, GP may also directly interact with γ-GCS, GS, GR or SOD enzymes to increase catalytic activities. All the effects of GP may contribute to increased GSH content and SOD activity in the SNc, which inhibits oxidative damage to lipids, proteins and DNA.

In conclusion, this study has demonstrated for the first time that GP appears to be able to protect the SNc against oxidative injury in an MPTP-induced mouse model of PD. Further studies are needed to investigate the molecular mechanism of the antioxidative effect of GP.

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Conflicts of interest
The authors had no conflicts of interest to declare in relation to this article.
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