

Full-length article

Ginsenoside Rg1 reduces MPTP-induced substantia nigra neuron loss by suppressing oxidative stress¹Xiao-chun CHEN², Yi-can ZHOU, Ying CHEN, Yuan-gui ZHU, Fang FANG, Li-min CHEN*Fujian Institute of Geriatrics, Union Hospital, Fujian Medical University, Fuzhou 350001, China***Key words**ginsenoside Rg1; *N*-acetylcystein; apoptosis; JNK mitogen-activated protein kinases; inbred C57BL mice

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Abstract

Aim: To investigate the effect of ginsenoside Rg1, an effective ingredient from ginsenoside, on 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced substantia nigra neuron lesion. **Methods:** C57-BL mice were given MPTP to prepare Parkinson disease mice model. Different doses of Rg1 (5, 10, and 20 mg·kg⁻¹·d⁻¹) or *N*-acetylcystein (NAC) (300 mg·kg⁻¹·d⁻¹) were given 3 d prior to MPTP in the pretreatment groups. Glutathione (GSH) level and total superoxide dismutase (T-SOD) activity in substantia nigra were determined by spectrophotometry. Nissl staining, tyrosine hydroxylase immunostaining, and TUNEL labeling were used to observe the damage and apoptosis of nigral neurons. Western blot analysis was used to detect the phospho-JNK and phospho-c-Jun levels in midbrain homogenates. **Results:** Pretreatments of C57-BL mice with different doses of Rg1 or NAC were found to protect against MPTP-induced substantia nigra neurons loss. Rg1 or NAC prevented GSH reduction and T-SOD activation in substantia nigra, and attenuated the phosphorylations of JNK and c-Jun following MPTP treatment. **Conclusion:** The antioxidant property of Rg1 along with the blocking of JNK signaling cascade might contribute to the neuroprotective effect of ginsenoside Rg1 against MPTP.

Introduction

The pathological hallmark of Parkinson disease (PD) is the loss of dopaminergic neurons in the substantia nigra (SN), which leads to the major clinical and pharmacological abnormalities that characterize the disease. Although the exact cause of neuronal loss is still unknown, current evidence points to the presence of ongoing oxidative stress and the generation of radical oxygen species (ROS) selectively in the pars compacta of substantia nigra (SNPC) of parkinsonian brains^[1, 2]. Human postmortem studies also suggested that oxidative damage to lipids, proteins, and DNA occurred in the SNPC of PD patients^[3–5]. The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is metabolized in the brain by monoamine oxidase-B to its toxic metabolite, 1-methyl-4-phenylpyridinium ion (MPP⁺), which is then selectively taken up into the dopaminergic neurons. MPP⁺ exerts its toxic effect in the dopaminergic neurons by inhib-

iting mitochondrial NADH dehydrogenase (NADH-DH)^[6]. MPTP could induce PD-like symptoms in humans and primates and causes degeneration of dopaminergic neurons in several animal species. The MPTP animal model is the ideal PD model for studying the pathogenesis of the disease.

The c-Jun NH₂-terminal kinase (JNK) signaling cascade has been implicated as a mediator of MPTP-induced apoptotic neuronal death^[7, 8]. Oxidative stress is an important activator of the JNK signaling cascade. In differentiated PC12 cells and cultured sympathetic neurons, increases in oxidative stress induced by antisense inactivation of superoxide dismutase led to increased JNK activity^[9].

Current treatments of PD are symptomatic and lack neuroprotective effect. Antioxidants have been focused on as candidates for the future treatment of PD. Ginsenoside Rg1, one of the active ingredients of ginseng, has potential neurotrophic and neuroprotective effects^[10]. In our previous study, we found Rg1 could protect substantia nigra neu-

rons against MPTP-induced apoptosis^[11], and in a *in vitro* study we revealed that Rg1 could suppress intracellular oxidative stress to prevent PC12 cells from dopamine-induced apoptosis^[12]. The aim of the current study is to further investigate whether Rg1 could suppress oxidative stress and block the activation of the JNK signaling cascade, and therefore protects dopaminergic neurons from apoptosis in an MPTP animal model. One antioxidant *N*-acetylcystein, which has been proved to protect against MPTP-induced substantia nigra neurons loss^[13], was used as a positive contrast in this study.

Materials and methods

Animals and treatment Male C57-BL mice weighing 18-22 g were divided into six groups ($n=8$). MPTP (Sigma, St Louis, MO, USA) was dissolved in saline at concentrations of 4 g/L. Ginsenoside Rg1 (purity >98 %), obtained from the Department of Organic Chemistry of Norman Bethune Medical University (Changchun, Jilin, China), was also dissolved in saline at concentrations of 1 g/L. Model animals were injected subcutaneously with saline for 3 d, followed by injection (subcutaneously) of MPTP (30 mg·kg⁻¹·d⁻¹) for 5 consecutive days. Three preventive groups were injected (ip) with Rg1 (5 mg·kg⁻¹·d⁻¹, 10 mg·kg⁻¹·d⁻¹, 20 mg·kg⁻¹·d⁻¹). Positive contrast group was injected (sc) with NAC (Jingke, Beijing, China, 300.0 mg·kg⁻¹·d⁻¹) for 8 days, and from the fourth day both Rg1 preventive groups and the positive contrast group were received injection (sc) of MPTP (30 mg·kg⁻¹·d⁻¹, 2 h after Rg1 or NAC injection) for 5 consecutive days. The control group received saline only. The animals were killed 24 h after the last injection. The mice were anaesthetized with euthanal and perfused transcardially with ice-cold saline prior to decapitation. The midbrain containing the substantia nigra was dissected, and quickly frozen at -80 °C until ready for later analysis. For immunohistochemistry analysis, the midbrain was fixated in 4 % formal-dehydum polymerisatum-PB and imbedded in paraffin. Serial 5- μ m-thick sections were cut transversely from the middle of the midbrain.

Spectrophotometry Substantia nigra were homogenized with 0.01 mol/L Tris-HCl (pH 7.4) consisting of 0.01 mol/L sucrose and 0.1 mmol/L EDTA, followed by centrifugation at 4000×g for 15 min. The supernatant was saved and analyzed. The dithio-binitrobenzoic acid method and xanthine/xanthine oxidase method were used to test glutathione (GSH) level and total superoxide dismutase (T-SOD) activity. Reaction product was determined by spectrophotometry. Test Kits were purchased from Jian Cheng Bioengineering Insti-

tute (Nanjing, China).

TUNEL Deparaffinized and hydrated sections were incubated with proteinase K (20 mg/L in 10 mmol/L Tris/HCl, pH=7.4-8.0) for 30 min at 37 °C, then incubated with TdT reaction mixture and AP-conjugated anti-fluorescein antibody (TUNEL detection kit, BM, Germany) for 1 h at 37 °C. Finally sections were visualized with NBT-BCIP. The nuclei of positive cells were stained blue-black.

Nissl staining Paraffin sections were deparaffinized and hydrated, stained with methylene blue buffer for 10 min and then immersed into acetic acid buffer for 2 min.

Immunohistochemistry Paraffin sections were deparaffinized and hydrated, then incubated with a goat anti-mouse TH polyclonal antibody (1:40 dilution in 0.01 mmol/L phosphate buffer saline, pH 7.4) at 4 °C overnight. Slides were then incubated with a biotinylated rabbit anti-goat IgG and SABC-reagent (SABC kit, BOSTER, Wuhan, Hubei, China), respectively for 30 min at 37 °C. At last, sections were stained with diaminobenzidine (DAB). Hemotoxylin was used to counterstain nuclei for one minute.

Western blot analysis Substantia nigra was homogenized (50 g/L) in lysis buffer (10 mmol/L Tris, 50 mmol/L NaCl, 0.03 μ mol/L sodium pyrophosphate, 50 mmol/L sodium fluoride, and 1% Triton X-100) containing 1 mmol/L phenylmethylsulfonyl fluoride, 20 mg/L aprotinin, and 1 mmol/L sodium vanadate^[14]. After homogenization, samples were centrifuged at 15 000×g for 15 min. The supernatants were taken for further analysis. Protein concentration was determined by the Bradford method^[15]. Approximately 10 μ g of total protein was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes were probed with polyclonal anti-p-JNK1/2 or anti-p-c-Jun rabbit antibody (Cell Signaling, Beverly, MA, USA). Immunoreactive bands were visualized by the addition of horseradish peroxidase-conjugated antibodies against rabbit Fab and chemiluminescent substrates (Pierce, Rockford, IL, USA), according to the manufacturer's instruction.

Image analysis The numbers of Nissl, TH, TUNEL-positive neurons in full microscopic fields of SN were counted with a digital medical image analysis system (Motic, Beijing, China). Three tissue sections per animal were counted for each index.

Data analysis Data were expressed as means±SEM. TUNEL-positive cells were calculated as: [TUNEL-positive cells/(TUNEL-positive cells+Nissl staining cells)]×100%. For statistical evaluation one-way analysis of variance (ANOVA) was employed. Student Newman Keuls test was performed when variance was equal, and Games-Howell test

was performed when variance was not equal. *Pearson* correlation analysis was also carried out to some indices. Statistical difference was considered to be significant only if $P < 0.05$.

Results

Effect of ginsenoside Rg1 on MPTP-induced apoptosis of substantia nigra neurons MPTP clearly had a detrimental effect on dopaminergic neurons. Treatment with MPTP decreased the numbers of TH-positive and Nissl-positive neurons and increased the percent of TUNEL-positive neurons in SN. Pretreatment with ginsenoside Rg1 or NAC significantly ameliorated the neuronal loss and apoptosis induced by MPTP ($P < 0.01$) (Figure 1–3). Rg1 at 10 mg/kg showed the best protective effect among all the doses but made no significant difference ($P > 0.05$) at 5 mg/kg and 20 mg/kg, which suggested that the effect of ginsenoside Rg1 might have an optimal dose range. There was no significant difference between NAC and Rg1 (10 mg/kg) treatment group.

Effect of ginsenoside Rg1 on GSH level and T-SOD activity in SN Decreased GSH level and increased T-SOD activity were detected in the substantia nigra of MPTP-treated mice. Both changes were attenuated by pre-administration with ginsenoside Rg1 or NAC (Table 1, $P < 0.01$). Better preventive effects were observed at doses of 10 and 20 mg/kg than at 5 mg/kg among Rg1 treated groups, but there was no significant difference among 10 mg/kg, 20 mg/kg of Rg1, and NAC (300.0 mg/kg). Correlation analysis showed a positive correlation between the positive neuron ratio of TH and the GSH level ($r = 0.885$, $P < 0.01$), and negative correlation between the positive neuron ratio of TH and the T-SOD activity ($r = -0.882$, $P < 0.01$). These results indicate that

enhanced oxidative stress was involved in the neurotoxicity of MPTP; and suitable doses of Rg1 could attenuate the oxidative stress induced by MPTP, while overdoses of Rg1 did not show better preventive effect.

Influence of MPTP-induced JNK and c-Jun phosphorylation by ginsenoside Rg1 MPTP treatment increased the levels of p46 and p54 phospho-JNK protein, as compared with the control. Preadministration of Rg1 or NAC partially attenuated the MPTP-mediated elevation in phospho-JNK and phospho-c-Jun levels in midbrain homogenates. A 10 mg/kg dose of Rg1 showed the greatest inhibition as did NAC, but the weak inhibition was observed at the dose of 20 mg/kg. These results suggested that Rg1 attenuated MPTP-induced phosphorylation of JNK and c-Jun, but a higher dosage was detrimental.

Discussion

Ginseng has been used in traditional Chinese medicine to enhance stamina and capacity to deal with fatigue and physical stress for thousands of years. Ginsenoside Rg1 is one of the active ingredients of ginseng. Previous *in vitro* studies showed that Rg1 could save cell from oxidative stress induced-apoptosis^[16]. In the current study, we observed that TH-positive neurons loss in SN caused by MPTP could be attenuated by Rg1 or NAC pretreatment; the result showed the neuronal protective effect of ginsenoside Rg1 against MPTP. Our study also revealed the neuroprotective mechanisms of Rg1 in preventing MPTP-induced loss of dopaminergic neurons in SN. The neurotoxin of MPTP is dependent on the inhibition of Complex I in the mitochondrial respiratory chain by MPP^+ , subsequently followed by ATP depletion and increased production of ROS^[17], eventually leads to the cell death. As one of the most important antioxidant molecule, GSH could clear H_2O_2 and prevent its reaction with iron to form the highly reactive OH radical in the Fenton reaction. Following MPTP treatment, GSH was consumed and its level dropped^[18,19]. We also observed the activity of T-SOD (including Cu/Zn-SOD and Mn-SOD), another important radical scavenger, was elevated in SN of MPTP treated mice; this change corresponded with those observed in PD patients^[20–22]. NAC is a free radical oxygen scavenger. Furthermore, it can be converted into cysteine after entering cells, and is an important substrate for GSH production. In our current study, pretreatment with NAC could attenuate the loss of GSH and the increased activity of T-SOD following MPTP treatment, same changes were observed among Rg1 pretreatment groups. We found that the antioxidative effect of Rg1 had an optimal dose of 10 mg/kg, this dosage

Table 1. Effect of ginsenoside Rg1 on GSH level and T-SOD activity. Mean \pm SEM. $n=8$. ^c $P < 0.01$ vs control group. ^f $P < 0.01$ vs MPTP group. ⁱ $P < 0.01$ vs MPTP+Rg1 5 mg/kg group.

Group	GSH level/ mg·mg ⁻¹ protein	SOD activity/ NU·mg ⁻¹ protein
MPTP	35.30 \pm 0.81 ^c	142.42 \pm 1.0 ^c
Control	56.96 \pm 0.41	116.53 \pm 1.03
MPTP+NAC(300 mg/kg)	56.18 \pm 0.74 ^{fc}	118.38 \pm 0.85 ^{fi}
MPTP+Rg1(5 mg/kg)	40.36 \pm 0.53 ^f	131.85 \pm 0.79 ^f
MPTP+Rg1(10 mg/kg)	50.09 \pm 0.48 ^{fi}	120.90 \pm 0.89 ^{fi}
MPTP+Rg1(20 mg/kg)	50.44 \pm 0.36 ^{fi}	122.84 \pm 0.73 ^{fi}

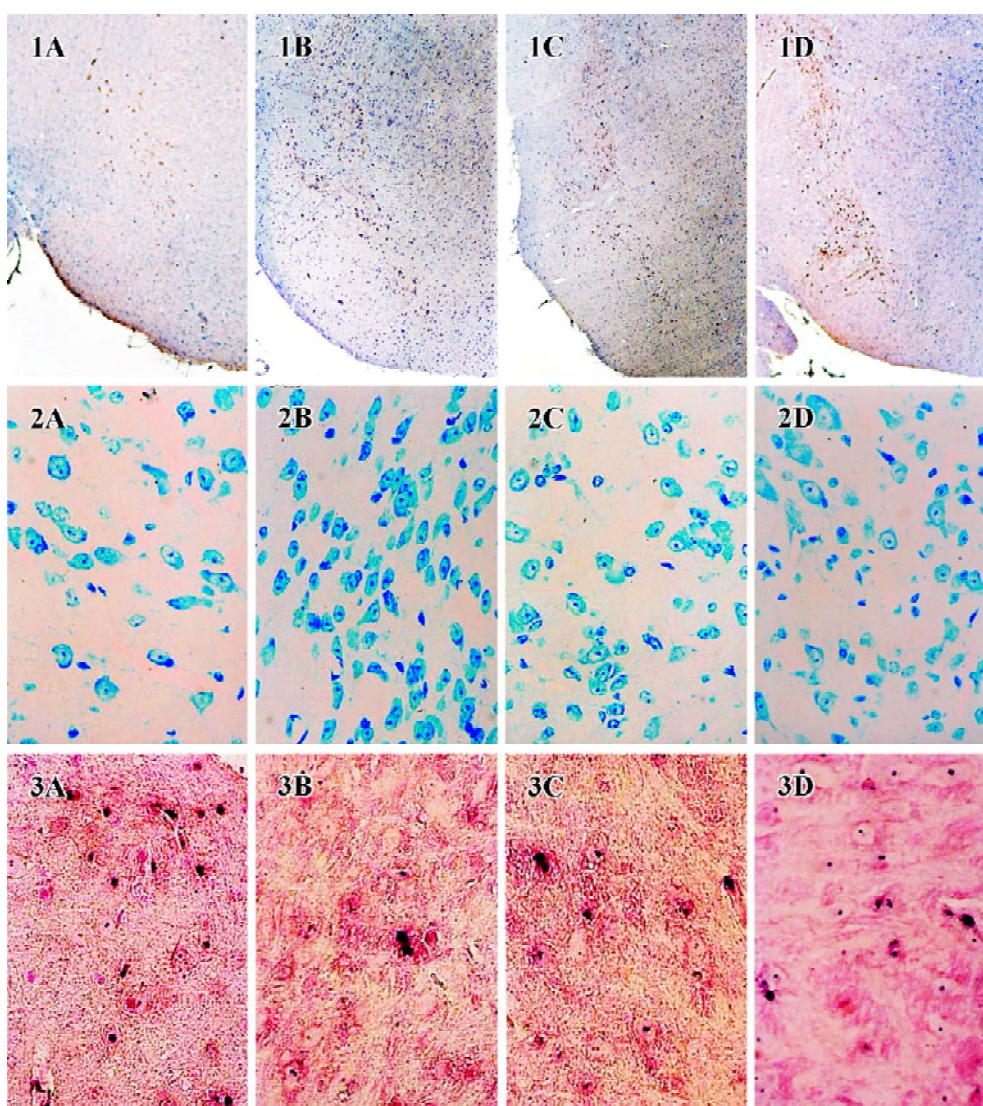


Figure 1. TH immunostaining (1), Nissl staining (2), and TUNEL staining (3) of mouse substantia nigra neurons. A: model group; B: control group; C: pretreatment with NAC group at 300.0 mg/kg; D: pretreatment with Rg1 group at 10.0 mg/kg; 1: $\times 40$. 2, 3: $\times 400$.

of Rg1 showed the similar effect as NAC, but there was no improvement beyond this dose. The results demonstrated that Rg1 possessed the same antioxidant property as NAC.

There has been no direct evidence indicating that Rg1 could scavenge radical by itself, therefore we tried to investigate whether Rg1 act as an antioxidant through mediator. In our previous study, we found the protective effect of Rg1 against MPTP-induced nigral neurons apoptosis was related to enhancing Bcl-2 expression and reducing iNOS expression^[11]. Bcl-2 is an anti-apoptotic protein located in the outer mitochondrial membrane, nuclear membrane and endoplasmic reticulum membrane. Bcl-2 participates in the regulation of cell death through multiple mechanisms, one of which

regulates the steady state of cellular ROS levels. This can be achieved either by modulating their production, usually by the mitochondria, or by affecting the antioxidant capacity of the cells. *In vitro* and *in vivo* studies provided evidences that Bcl-2 could attenuate cell injuries caused by oxidative stress. Bcl-2 could protect against the increased level of ROS caused by dopamine metabolism in PC12 cells^[23]. Transfection with Bcl-2 provided the PC12 cells with marked protection against dopamine-induced apoptosis and inhibited dopamine oxidation to dopamine-melanin^[24]. Further studies demonstrated enhanced oxidative stress and susceptibility to oxidants in the brains of Bcl-2-deficient mice^[25]. Our previous study showed that Rg1 could prevent

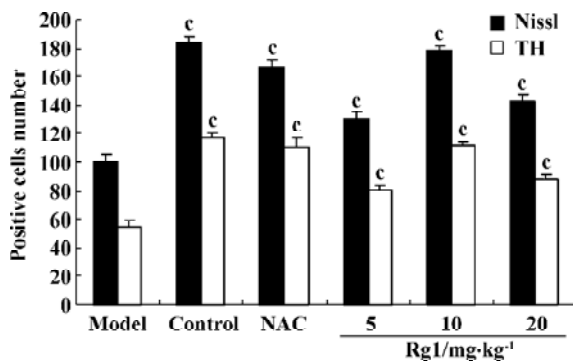


Figure 2. Number of Nissl (black) and TH (white) staining-positive neurons in SN. Pretreatment with Rg1 significantly increased the number of survival neurons and TH-positive neurons, as determined by one-way ANOVA. A 10 mg/kg dose of Rg1 showed the best protective effect among all the doses, and showed no significant difference with NAC. $n=8$. Mean \pm SEM. ^c $P<0.01$ vs the model group.

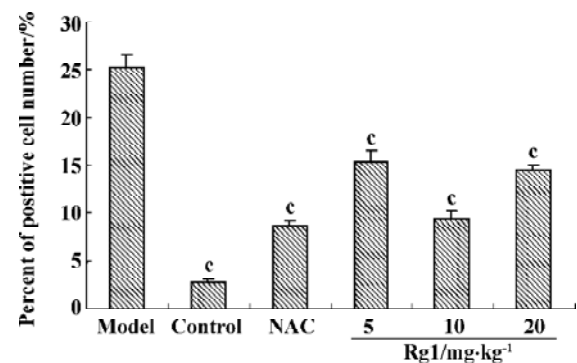


Figure 3. Percentage of TUNEL-positive neuron numbers in SN. The percentage of TUNEL-positive neurons in the model group increased significantly compared with other groups. Pretreatment with Rg1 significantly decreased the percentage of TUNEL-positive neurons. A 10 mg/kg dose of Rg1 showed the best preventive effect of all the doses, and showed no significant difference with NAC. $n=8$. Mean \pm SEM. ^c $P<0.01$ vs the model group.

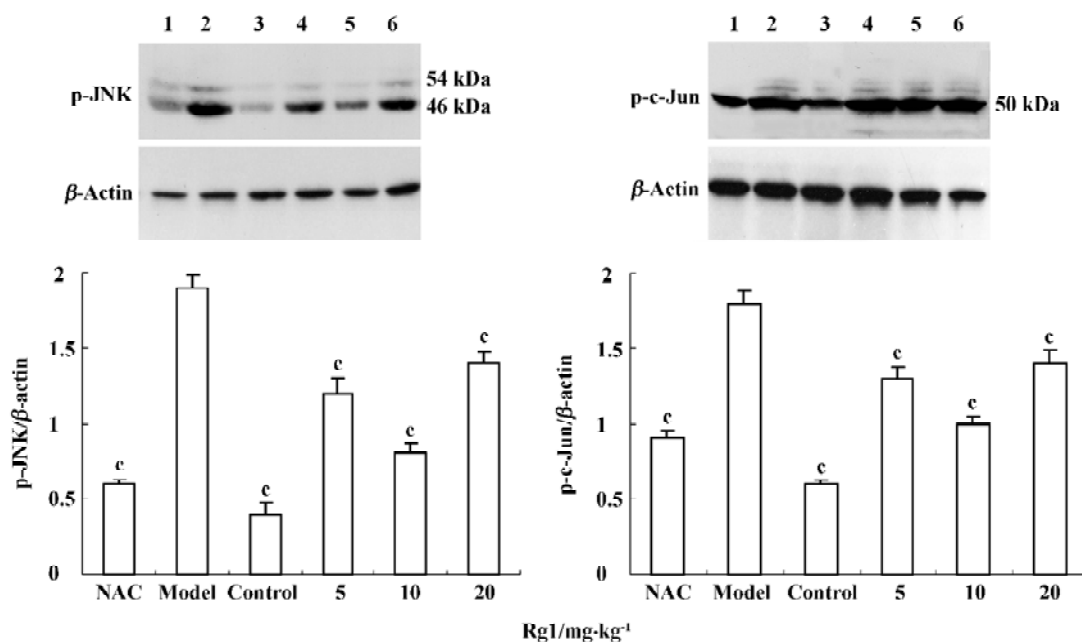


Figure 4. Western blot detection of phospho-JNK and phospho-c-Jun levels respectively. The histogram showed the comparisons of OD odds among different groups. 1: NAC; 2: model; 3: control; 4: 5 mg/kg Rg1+MPTP; 5: 10 mg/kg Rg1+MPTP; 6: 20 mg/kg Rg1+MPTP. $n=3$. Mean \pm SEM. ^c $P<0.01$ vs the MPTP group.

the decrease of Bcl-2-positive cell numbers following MPTP treatment, so this might contribute to the antioxidant property of Rg1.

Another important protein involved in oxidative stress is nitric oxide synthase (NOS), of which three isoforms have been described: neuronal NOS (nNOS) and endothelial NOS

are calcium-calmodulin-dependent enzymes, while inducible NOS (iNOS) activity is independent of intracellular calcium levels. Nitric oxide (NO), an important free radical generated by NOS, can react with superoxide radicals to form the oxidizing agents peroxynitrite (ONOO⁻) and OH⁻ [26], and eventually induce DNA strand breakage and apoptosis [27].

Numerous experimental studies have shown that iNOS play an important role in MPTP-induced dopaminergic neuron injury or in PD patients^[28,29], it is in agreement with our previous observation that MPTP increased the numbers of iNOS-positive cells. Therefore, changes of Bcl-2 and iNOS expressions could play important roles in the antioxidative effect of Rg1, this could explain the antioxidant property of Rg1. However, the exact pathway leading to the changes of Bcl-2 and iNOS expression in SN is still unclear.

The JNK signaling cascade is a main pathway that mediates the neurotoxicity of MPTP^[14], CEP-1347/KT-7515, an inhibitor of JNK activation, can attenuate MPTP-induced nigrostriatal dopaminergic degeneration^[7]. Transgenic mice expressing the JNK binding domain (JBD) of JNK interacting protein 1 could resist the neurotoxicity of MPTP^[8]. Oxidative stress is an important activator of the JNK signaling cascade. In differentiated PC12 cells and cultured sympathetic neurons, oxidative stress induced by antisense inactivation of superoxide dismutase increased JNK activity^[9]. In our previous study, we observed that JNK pathway was activated after the exposure of SHSY5Y cells to the mitochondrial complex I inhibitor MPP⁺, Rg1 could attenuate the elevation of ROS and reduce the JNK activation after MPP⁺ treatment^[30]. In the present *in vivo* study, we also found that pretreatment with Rg1 or NAC could attenuate the elevated phosphorylation of JNK and c-Jun induced by MPTP in SN. The dose of 10 mg/kg of Rg1 showed better effect than dose of 5 mg/kg and 20 mg/kg. There was no significant difference between NAC and 10 mg/kg Rg1, but it is a puzzle why 20 mg/kg Rg1 had weaker inhibition than 10 mg/kg Rg1. The toxicity of higher doses of Rg1 could compromise its ability as a radical scavenger. However, this change is associated with the loss of dopaminergic neurons at different Rg1 doses. This results confirms previous studies that JNK signaling cascade play an important role in dopaminergic neuron apoptosis induced by MPTP, and appropriate dose of Rg1 could attenuate the MPTP induced activation of JNK signaling cascade. It requires further study to find out whether Rg1 influences JNK pathway through mechanisms other than antioxidation.

In conclusion, the present study indicates that pretreatment with certain doses of Rg1 could reduce the loss of dopaminergic neurons caused by MPTP in SN. The neuroprotective effects of Rg1 are potentially due to its antioxidant property that involves Bcl-2 and iNOS, and its inhibitory effect on JNK signaling cascade activation. Further studies will be carried out to evaluate whether ginsenoside Rg1, an important ingredient of ginsenoside, could benefit as a future preventive and therapeutic drug of PD.

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