ISSN 1671-4083
Shanghai Institute of Materia Medica
Chinese Academy of Sciences
http://www.ChinaPhar.com

Protective effect of ginsenoside Rg1 against MPTP-induced apoptosis in mouse substantia nigra neurons[1]

CHEN Xiao-Chun[2], CHEN Ying, ZHU Yuan-Gui, FANG Fang, CHEN Li-Min

Fujian Institute of Geriatrics, Union Hospital, Fujian Medical University, Fuzhou 350001, China

KEY WORDS ginseng; saponins; apoptosis; Parkinson disease; animal disease models

ABSTRACT

AIM: To explore the possible mechanism of the ginsenoside Rg1 in protecting substantia nigra neurons from 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP)-induced apoptosis in C57BL mice. **METHODS:** C57BL male mice were given with MPTP to prepare Parkinson's disease mouse model. Different doses of Rg1 (2.5, 5.0, and 10.0 mg/kg, respectively) were given 3 d prior to MPTP in the pretreatment groups. Nissl staining, TH immunostaining, and TUNEL labeling were used to observe the damage and apoptosis of nigral neurons. The immunohistochemistry assay was used to detect the protein levels of Bcl-2, Bcl-xl, Bax, inducible nitric oxide synthase (iNOS), neuronal NOS (nNOS), and cleaved caspase-3. **RESULTS:** Compared with MPTP model group, pretreatment with Rg1 (5.0 and 10.0 mg/kg) was shown to increase the Nissl staining neurons and TH-positive neurons ($P<0.01$), and to decrease the TUNEL-positive neurons in the substantia nigra zona compacta ($P<0.01$). Moreover, Rg1 elevated the levels of cleaved caspase-3, Bax, and iNOS, but reduced the levels of Bcl-2 and Bcl-xl ($P<0.01$). **CONCLUSION:** Rg1 has protective effect against MPTP-induced apoptosis and this effect may be attributed to enhancing Bcl-2 and Bcl-xl expression, reducing Bax and iNOS expression, and inhibiting activation of caspase-3.

INTRODUCTION

Apoptosis, also called programmed cell death, as a genetically regulated cell death process, may underlie the neuron-specific degeneration and has gathered great attention in recent years. MPTP is a neurotoxin that has been extensively used in various animal species to model the symptoms and pathology of Parkinson's disease (PD)[1]. By elucidating the mechanism of MPTP-toxicity, Tatton and Kish[2] had reported that apoptotic cell death was observed in the MPTP mouse model induced by small doses of MPTP, given over a prolonged period of time.

Ginsenoside Rg1 was shown to be useful in the

[1] Project supported by Fujian Science and Technology Foundation, № 2001 Z 037.
[2] Correspondence to Prof CHEN Xiao-Chun.
Phn 86-591-337-7664. Fax 86-591-334-0575.
E-mail ZJCXCH@Public5.FJ.FZ.cn
Received 2001-12-24 Accepted 2002-05-10

alleviation of the symptoms of some senile diseases[3]. Recently, studies showed it prevented rat cortical neurons from apoptosis[4]. Our previous study had also reported the protective effect of ginsenoside Rg1 on dopamine (DA)-induced apoptosis of PC12 cells, an *in vitro* model system of PD[5].

In the present study, we studied whether Rg1 could prevent substantia nigra neurons from MPTP-induced apoptosis in C57BL mice and the possible mechanism of this process.

MATERIALS AND METHODS

Animal model and treatment Thirty eight-week-old male C57BL mice, weighing 20 g±2 g, were divided into five groups (n=6). The model group was injected ip with MPTP (Sigma) 30 mg·kg^{-1}·d^{-1} for 5 d, while the control group was injected with equal dose of saline, and the pretreatment groups 1, 2, and 3 were pretreated with Rg1 (2.5, 5.0, or 10.0 mg·kg^{-1}·d^{-1}, ip, respectively for 3 d) before MPTP injection and then in the following 5 d these groups were treated with Rg1 once again (the doses were mentioned above) 2 h before receiving MPTP injection as mentioned.

Tissue preparation All mice were killed 24 h after the last injection and perfused transcardially with 4 % paraformaldehyde in phosphate buffer (PB, 0.1 mmol/L, pH 7.4). The substantia nigra zona compacta (SNc) of mouse was isolated according to the standard stereotaxic atlas of mouse brain and immersion-fixed in 4 % paraformaldehyde in PB 0.1 mmol/L (pH 7.4) for 2 h at 4 ℃. Following dehydration in PB (pH 7.4) supplemented with 20 % sucrose, serial coronal sections were cut through SNc at 10 μm on a cryostat (Leica, Germany). The sections were mounted on slides and stored at -80 ℃ until used.

Nissl staining Sections were stained with methylene blue buffer for 10 min and then put into acetic acid buffer for 2 min. The plasma of stained neuron is blue.

Tyrosine hydroxylase (TH) immunohistochemistry Sections were incubated with 0.3 % Triton X-100 in PB 0.1 mmol/L for 1 h at room temperature, and then incubated with a goat anti-mouse TH polyclonal antibody (1:40 dilution in 0.01 mmol/L phosphate buffer saline, pH 7.4) overnight at 4 ℃. Slides were then incubated in order with a biotinylated rabbit anti-goat IgG and SABC-reagent (SABC kit, Boster Biology Technique Company, Wuhan, China) for 30 min at 37 ℃. At last, sections were visualized with diaminobenzidine (DAB). The plasma and process of positive cell were stained brown.

Detection of apoptotic cells by TUNEL Sections were incubated with 0.1 % Triton X-100 in 0.1 % sodium citrate for 1 h at room temperature, and then incubated in order with TdT reaction mixture and AP-conjugated anti-fluorescein antibody (TUNEL detection kit, BM, Germany) for 30 min at 37 ℃. At last, sections were visualized with NBT-BCIP. The nuclei of positive cells were stained blue-black.

Immunohistochemistry of Bcl-2, Bax, Bcl-xl, cleaved-caspase-3, iNOS, and nNOS The primary antibodies were rabbit anti-mouse polyclonal antibodies, respectively against Bcl-2 (1:1000, R&D, USA), Bax (1:500, R&D, USA), Bcl-xl (1:40, Santa Cruz, USA), cleaved caspase-3 (M_r 17 000-20 000, 1:50, Cell signaling, USA), iNOS (1:40, Santa Cruz, USA) and nNOS (1:40, Santa Cruz, USA). The plasma of positive cells was stained brown.

Data analysis Data were expressed as mean ±SD. TUNEL-positive percent was calculated as: [TUNEL-positive cells/(TUNEL-positive cells+Nissl staining cells)]×100 %. The positive percents of Bcl-2, Bcl-xl, Bax, caspase-3 and NOS were calculated as: (positive cells/Nissl staining cells)×100 %. For statistical evaluation one-way analysis of variance (ANOVA) were 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 performed to some index. Statistical significance was assumed at P<0.05.

RESULTS

Protective effect of Rg1 on MPTP-induced apoptosis of substantia nigra neurons The counts of Nissl staining neurons and TH-immunoreactive (IR) neurons in the SNc were significantly decreased, and

TUNEL-positive percent was significantly increased by MPTP-treatment compared with saline injections. However, pretreatment with Rg1 (5.0, 10.0 mg/kg) significantly antagonized MPTP-induced loss of Nissl staining and TH-IR neurons, and significantly decreased TUNEL-positive percent [Fig 1(1, 2) and Fig 2].

Effects of Rg1 on expression of Bcl-2, Bax, and Bcl-xl The percents of Bcl-2 and Bcl-xl immunoreactive cells in the SNc were decreased markedly and the percent of Bax-IR cells was increased markedly by MPTP-treatment compared with saline injections. On the contrary, after pretreatment with Rg1, the percents of Bcl-2 (the pretreatment group 2 and 3) and Bcl-xl-IR (the pretreatment group 3) cells were increased markedly, but the percent of Bax-IR cells (the pretreatment group 2 and 3) was decreased markedly [Tab 1

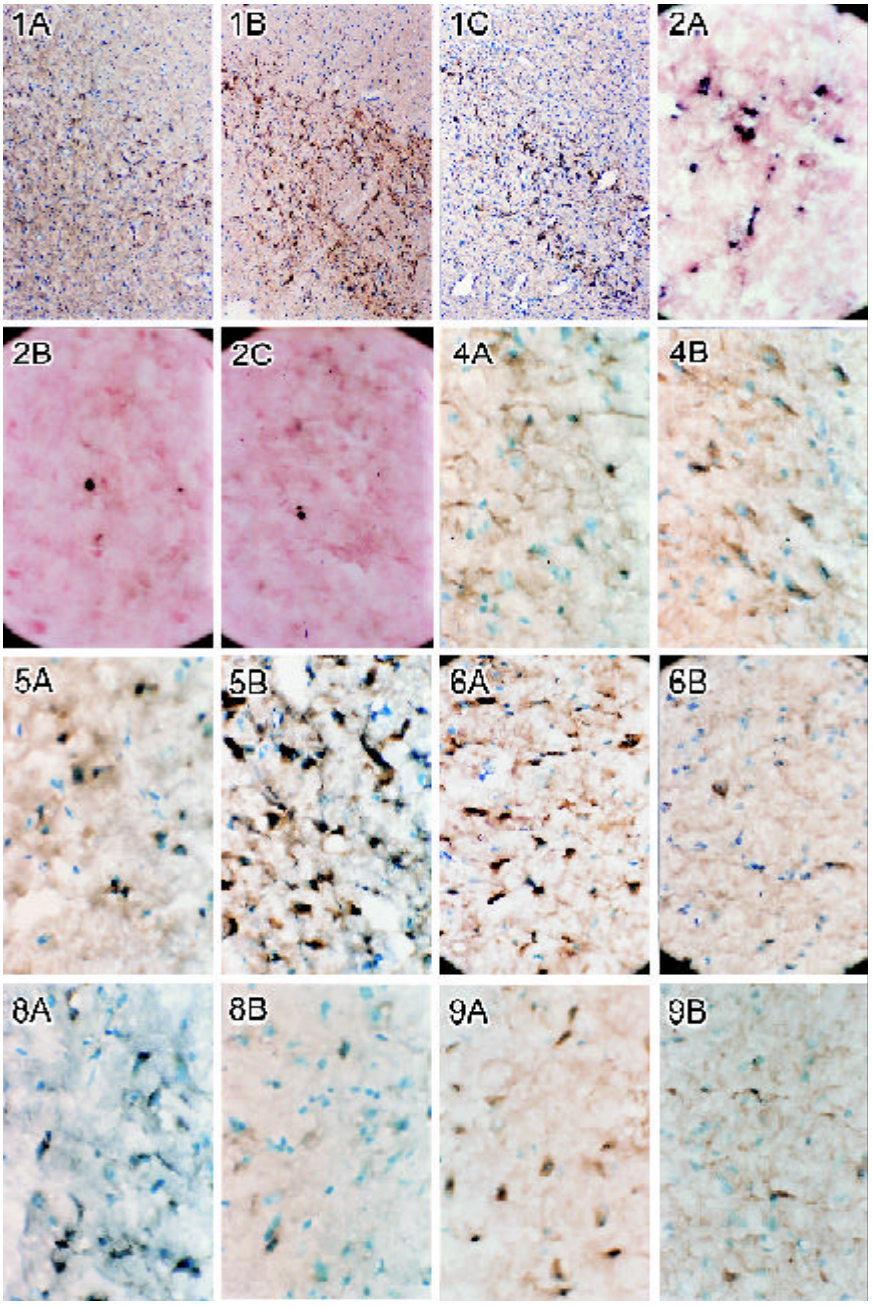

Fig 1. TH (1), Bcl-2 (4), Bcl-xl (5), Bax (6), cleaved caspase-3 (8) and iNOS (9) immunostaining and TUNEL (2) staining of mouse substantia nigra neurons. A: model group; B: pretreatment with Rg1 group at 10.0 mg/kg; C: control group. (1): ×100, others ×400.

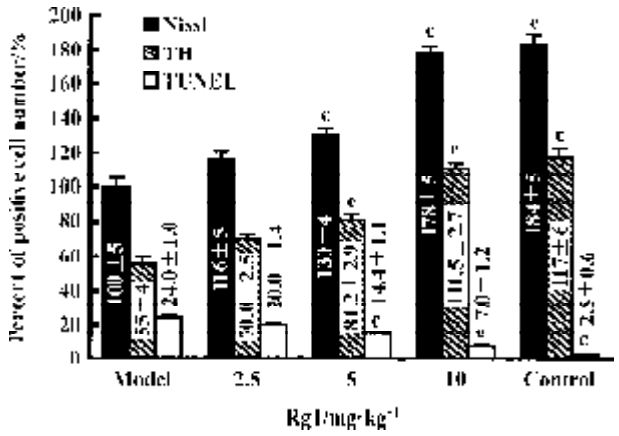

Fig 2. Nissl, TH, TUNEL staining in the mouse SNc neurons. The Nissl and TH positive cell number was markedly increased, but TUNEL-positive percent was markedly decreased, in the SNc of control group and pretreatment group (Rg1 at 5.0, 10.0 mg/kg), compared with that of model group. ^cP <0.01 *vs* model.

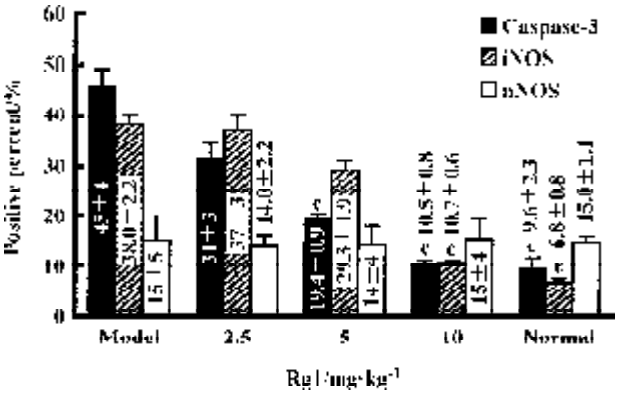

Fig 3. Cleaved caspase-3, iNOS, nNOS staining in the mouse SNc neurons. The cleaved caspase-3 positive percent and iNOS-positive percent was markedly decreased, but nNOS-positive percent has no change, in the SNpc of normal group and pretreatment group (Rg1 at 5.0,10.0 mg/kg), compared with that of model group. ^cP<0.01 *vs* model.

Tab 1. Expression of Bcl-2, Bcl-xl, and Bax in the SNc. *n*=6. mean±SD. ^bP<0.05, ^cP<0.01 *vs* model group.

Groups	Bcl-2 positive percent/%	Bcl-xl positive percent/%	Bax positive percent/%
Model group	15.2±1.5	23.7±1.1	39±3
Control group	24.1±1.7^c	32.6±1.7^c	11.9±0.8^c
Rg1 2.5 mg/kg	16.8±0.7	24.7±2.2	30.8±1.4^b
Rg1 5.0 mg/kg	34.2±1.8^c	29.2±2.3^b	27.3±1.4^c
Rg1 10.0 mg/kg	36.4±1.7^c	38.2±1.6^c	16.1±1.2^c

and Fig 1(4, 5, 6)].

Effect of Rg1 on caspase-3 activation After treatment with MPTP, the percent of cleaved caspase-3-IR cells in the SNc was markedly increased, while it was decreased greatly when pretreated with Rg1 5.0 or 10.0 mg/kg [Fig 1(8) and Fig 3].

Effects of Rg1 on expression of iNOS and nNOS After treatment with MPTP, the percent of iNOS-IR cells in the SNc was increased significantly. Pretreatment with Rg1 (10.0 mg/kg) decreased the percent markedly. The percent of nNOS-IR cells had no difference between the model group and pretreatment groups [Fig 1(9) and Fig 3].

Correlation of TUNEL-positive percent with Bcl-2, Bcl-xl, Bax, iNOS, and nNOS expression, and caspase-3 activation TUNEL-positive percent correlated negatively with positive percents of Bcl-2 and Bcl-xl (r=-0.559, -0.717, P<0.01). However, TUNEL-positive percent correlated positively with the positive percents of Bax, cleaved-caspase-3, and iNOS (r=0.867, 0.858, 0.888, P<0.01), and had no correlation with the percent of nNOS-IR cells (r=-0.078, P>0.05).

DISCUSSION

MPTP is a neurotoxin that can induce degeneration and death of dopaminergic neurons, and apoptosis may be involved in this kind of cell death. The present study showed that MPTP could induce the apoptosis of dopaminergic neurons in the SNc, which indicated that C57BL mice treated with MPTP offered a substantial Parkinson's disease model to further research its pathogenesis and therapy. Ginsenoside Rg1, one of important components of ginseng, was shown to have a great potential neotropic and neurotrophic or neuroprotective effect recently[6,7]. In the present study, pretreatment with Rg1 (5.0, 10.0 mg/kg) was found to reduce the toxicity of MPTP to dopaminergic neurons in the SNc, suggested that Rg1 had potential protective

effect on MPTP-induced neurotoxicity.

It is true that Bcl-2 family proteins, as important regulators of apoptosis, include Bcl-2 and Bcl-xl (antiapoptotic member), and Bax(proapoptotic member)[8]. Overexpression of Bcl-2 in the SNc neurons could resist injuries by MPTP and 6-hydroxydopamine (6-OHDA)[9]. In the study of Hassouna *et al*[10], MPTP-treatment increased Bax expression in the SNc neurons, with which the present study was in agreement. The present study also showed that Bcl-2 and Bcl-xl expression were enhanced, and Bax expression was reduced in the SNc neurons in protective effects of Rg1 against MPTP-induced apoptosis. So, these results indicated that the increased expression of Bcl-2 and Bcl-xl and decreased expression of Bax may be important mechanisms in protective effects of Rg1 against MPTP-induced apoptosis.

Aspartate-specific cysteine proteases (caspase) are essential in the pathway of apoptotic death. Hartmann *et al*[11] found caspase-3 might be a vulnerable factor and effector of apoptotic death in dopaminergic neurons in the SNc of PD animal model and PD patients. The present study showed that caspase-3 activition was inhibited after pretreatment with Rg1 compared with MPTP model, indicating that Rg1 may inhibit activation of caspase-3 during attenuating MPTP-induced apoptosis.

Nitric oxide (NO) is one of free radicals and cell signaling molecule. It was reported that NO, as a toxic factor, could mediate the death of dopaminergic neurons[12]. Inducible NOS and neuronal NOS can catalyze *L*-arginine and synthesize NO. Tamatani *et al*[13] reported that neurons cocultured with astrocytes subjected to hypoxia undervent apoptotic death, and this process was dependent on the increased expression of iNOS and so overproduction of NO. Heneka *et al*[14] reported that the increased expression of iNOS proceeded apoptotic cell death of cerebellar granule neurons and this kind of NO-mediated apoptosis was accompanied with enhanced caspase-3 activity, moreover, the drug which down-regulated iNOS expression could attenuate apoptosis correspondingly. In the present study, Rg1 was shown to decrease the expression of iNOS, suggesting Rg1 could reduce NO production by decreasing iNOS expression in the process of protecting from MPTP-induced apoptosis.

In conclusion, the present study suggested that Rg1 could protect nigral neurons from MPTP-induced apoptosis through increasing the level of Bcl-2 and Bcl-xl expression, decreasing the level of Bax and iNOS expression, and inhibiting the activation of caspase-3.

REFERENCES

1 Chen SD, Xu DL, Yu HZ. Study on 1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine-induced Parkinson's disease animal model. Chin Med J 1990; 70: 252-4.

2 Tatton NA, Kish SJ. *In situ* detection of apoptotic nuclei in the substantia nigra compacta of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice using terminal deoxynucleotidyl transferase labelling and acridine orange staining. Neuroscience 1997; 77: 1037-48.

3 Zhang JT. Retrospect and prospect of Ginseng research. Acta Pharm Sin 1995; 30: 321-5.

4 Li JQ, Zhang XG, Zhang JT. Study on the anti-apoptotic mechanism of ginsenoside Rg1 in cultured cortical neurons. Acta Pharm Sin 1997; 32: 406-10.

5 Chen XC, Zhu YG, Wang XZ, Zhu LA, Huang C. Protective effects of ginsenoside Rg1 on dopamine-induced apoptosis in PC12 cells. Acta Pharmcol Sin 2001; 22: 673-8.

6 Wang XY, Zhang JT. Effects of ginsenoside Rg_1 on synaptic plasticity of freely moving rats and its mechanism of action. Acta Pharmacol Sin 2001; 22: 657-62.

7 Rudakewich M, Ba F, Benishin CG. Neurotrophic and neuroprotective actions of ginsenosides Rb (1) and Rg (1). Planta Med 2001; 67: 533-7.

8 Reed JC. Double identity for proteins of the bcl-2 family. Nature 1997; 387: 773-6.

9 Offen D, Beart PM, Cheung NS, Pascoe CJ, Hochman A, Gorodin S, *et al.* Transgenic mice expressing human Bcl-2 in their neurons are resistant to 6-hydroxydopamine and 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine neurotoxicity. Proc Natl Acad Sci USA 1998; 95: 5789-94.

10 Hassouna I, Wickert H, Zimmermann M, Gillardon F. Increase in bax expression in substantia nigra following 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) treatment of mice. Neurosci Lett 1996; 204: 85-8.

11 Hartmann A, Hunot S, Michel PP. Caspase-3: A vulnerability factor and final effector in apoptotic death of dopaminergic neurons in Parkinson's disease. Proc Natl Acad Sci USA 2000; 97: 2875-80.

12 Lavoie MJ, Hastings TG. Peroxynitrite- and nitrite-induced

oxidation of dopamine: implications for nitric oxide in dopaminergic cell loss. J Neurochem 1999; 73: 2546-54.

13 Tamatani M, Ogawa S, Nitsu Y, Tohyama M. Involvement of Bcl-2 family and caspase-3-like protease in NO-mediated neuronal apoptosis. J Neurochem 1998; 71: 1588-96.

14 Heneka MT, Feinstein DL, Galea E, Gleichmann M, Wullner U, Klockgether T. Peroxisome proliferator-activated receptor gamma agonists protect cerebellar granule cells from cytokine–induced apoptotic cell death by inhibition of inducible nitric oxide synthase. J Neuroimmunol 1999; 100: 156-68.

人参皂苷 Rg1 对小鼠 1- 甲基 -4- 苯基 -1,2,3,6- 四氢吡啶诱导的黑质神经元凋亡的保护作用[1]

陈晓春[2]，陈　滢，朱元贵，方　芳，陈丽敏
（福建医科大学附属协和医院福建省老年医学研究所，福州 350001，中国）

关键词　人参；皂苷类；细胞凋亡；帕金森病；动物疾病模型

目的：探讨人参皂苷 Rg1 对抗 1- 甲基 -4- 苯基 -1,2,3,6- 四氢吡啶（MPTP）诱导的 C57BL 小鼠黑质神经元凋亡的可能机制．方法：MPTP(30 mg·kg^{-1}·d^{-1} × 5 d)腹腔注射制备 C57BL 小鼠帕金森病模型，同时预防组分别以不同剂量人参皂苷 Rg1 (2.5、5.0、10.0 mg·kg^{-1}·d^{-1} × 8 d)于 MPTP 注射前预先腹腔注射小鼠．用 Nissl 染色和 TH 组化染色观察黑质损害情况，TUNEL 染色检测细胞凋亡，同时运用免疫组织化学方法检测 caspase-3 的活性片段以及 Bcl-2、Bcl-xl、Bax、iNOS 和 nNOS 的表达情况．结果：人参皂苷 Rg1 (5.0 和 10.0 mg/kg)预处理能使黑质致密带 Nissl 阳性神经元和 TH 阳性神经元的脱失减少，同时降低了 TUNEL 阳性率，并伴有 Bcl-2 和 Bcl-xl 表达增加，Bax 和 iNOS 表达减少以及抑制 caspase-3 的激活．结论：人参皂苷 Rg1 预处理对 MPTP 诱导的小鼠黑质神经元凋亡有明显的保护作用，其作用可能是通过降低 iNOS 和 Bax 蛋白表达，增加 Bcl-2 和 Bcl-xl 蛋白表达以及抑制 caspase-3 的激活来实现的．

（责任编辑　吴民淑）