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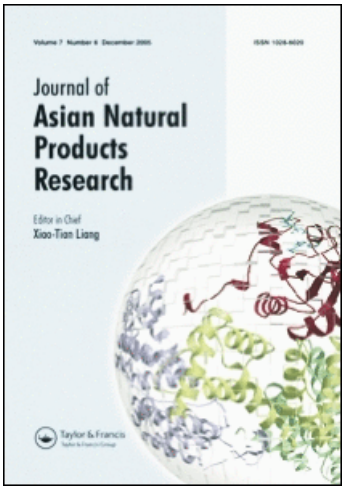

Journal of Asian Natural Products Research
Publication details, including instructions for authors and subscription information:
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Possible mechanisms of the protection of ginsenoside Re against MPTP-induced apoptosis in substantia nigra neurons of Parkinson's disease mouse model

Bei-Bei Xu[a]; Chun-Qing Liu[b]; Xi Gao[b]; Wan-Qin Zhang[b]; Shi-Wei Wang[b]; Ying-Lin Cao[a]
[a] Physiological Department, Shenyang Pharmaceutical University, Shenyang, Liaoning, China [b] Physiology Department, Dalian Medical University, Dalian, Liaoning, China

To cite this Article Xu, Bei-Bei , Liu, Chun-Qing , Gao, Xi , Zhang, Wan-Qin , Wang, Shi-Wei and Cao, Ying-Lin(2005) 'Possible mechanisms of the protection of ginsenoside Re against MPTP-induced apoptosis in substantia nigra neurons of Parkinson's disease mouse model', Journal of Asian Natural Products Research, 7: 3, 215 — 224
To link to this Article: DOI: 10.1080/10286020410001690172
URL: http://dx.doi.org/10.1080/10286020410001690172

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Journal of Asian Natural Products Research, Vol. 7, No. 3, June 2005, 215–224

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Possible mechanisms of the protection of ginsenoside Re against MPTP-induced apoptosis in substantia nigra neurons of Parkinson's disease mouse model

BEI-BEI XU†, CHUN-QING LIU‡, XI GAO‡, WAN-QIN ZHANG‡, SHI-WEI WANG‡ and YING-LIN CAO†*

†Physiological Department, Shenyang Pharmaceutical University, Shenyang, Liaoning 110016, China
‡Physiology Department, Dalian Medical University, Dalian, Liaoning, China

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(Received 24 November 2003; in final form 21 February 2004)

We have investigated the role of ginsenoside Re (Re) in preventing 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced apoptosis of the substantia nigra neurons in the mouse model of Parkinson's disease (PD). C57BL mice have been administrated i.s.c. with MPTP to establish the PD model. Pretreatment groups were given different doses of Re (6.5, 13, 26 mg kg^{-1}) i.g. for 13 days. Transmission electron microscope (TEM), tyrosine hydroxythase (TH) immunostaining and TDT-mediated dUTP nick-end labeling (TUNEL) staining have been used to observe the damage of substantia nigral neurons. To measure the expression of inducible nitric oxide synthase (iNOS), Bcl-2, Bax protein and expression of Bcl-2, Bax gene, immunohistochemistry and *in situ* hybridization have been explored respectively. Western blot analysis has been performed with anti-caspase-3. Pretreatment with Re (13, 26 mg kg^{-1}) markedly increases TH-positive neurons and decreases the TUNEL-positive ratio compared with the MPTP model group. Furthermore, Re could enhance the expression of Bcl-2 protein and *Bcl-2* mRNA, but reduce the expression of Bax, *Bax* mRNA, and iNOS, and weaken the cleavage of caspase-3. In summary, ginsenoside Re showed protection from MPTP-induced apoptosis in the PD model mouse nigral neurons and this effect may be attributable to upregulating the expression of Bcl-2 protein, downregulating the expression of Bax, and iNOS protein, and inhibiting the activation of caspase-3.

Keywords: Ginsenoside Re; Parkinson's disease; MPTP; Apoptosis; Substantia nigra neuron

1. Introduction

Apoptosis, or programmed cell death (PCD), is the process by which a cell will actively suicide under tightly controlled circumstances. Defective regulation of PCD may play a part in the etiology of degenerative diseases of the central nervous system (CNS). Parkinson's disease (PD) is characterized by the selective loss of dopaminergic neurons in the substantia nigra pars compact (SNc). Even after numerous studies, the cause of dopaminergic cell degeneration in SNc of PD patients has not been identified with certainty [1]. Therefore, several experimental models have been developed for PD; the most frequently used is that

*Corresponding author. Tel.: +86-24-23843711-3365. Fax: +86-24-83892186. E-mail: xupeipei76@sohu.com

Journal of Asian Natural Products Research
ISSN 1028-6020 print/ISSN 1477-2213 online © 2005 Taylor & Francis Ltd
http://www.tandf.co.uk/journals
DOI: 10.1080/10286020410001690172

produced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) administration in mice. Ziv *et al.* [2] have reported that the death of dopaminergic neurons in parkinsonism may be due to many causes, for instance free radical-mediated damage, the induction of glutamate-mediated excitotoxicity, mitochondria dysfunction and others factors, but it is possible that apoptosis underlies these factors. Many lines of evidence point to a significant contribution of apoptosis to cell death after application of 1-methyl-4-phenylpyridinium (MPP^+) in cell cultures or MPTP *in vivo* [3]. Thus, protecting neurons and anti-apoptosis have become the primary idea for curing Parkinson's disease.

Ginseng (*Panax ginseng*) has been used as a tonic in Chinese traditional medicine for 2000 years. It is very useful in restoring the deteriorated functions of aged persons and animals. Ginseng saponins (GS), as the effective component of Ginseng, possess extensive bioactivity, including anti-aging, anti-oxidation, anti-apoptosis, and anti-tumour, which has been variously and systematically explored [4]. Ginsenoside Re is one of the important active principles of ginseng and shares many pharmacological effects of this plant, but protection of Re against MPTP-induced apoptosis in Parkinson's disease mouse model has not been reported.

Here, we studied whether Re could prevent substantia nigra neurons from MPTP-induced apoptosis in C57BL mice of the PD model, along with the possible mechanism of action.

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2. Results and discussion

2.1 Protection of Re on apoptosis of dopaminergic neurons in SNc

As shown in figure 1, TEM revealed nuclear chromatin condensation with apoptotic features and neurons with condensed chromatin margination at the nuclear periphery associated with pericellular space formation created by cell shrinkage in pigmented neurons of MPTP model group. After treatment with MPTP, TH-positive neurons significantly decreased, and the TUNEL-positive percent markedly increased compared with the control group. However, pretreatment with Re (13, 26 mg kg^{-1}) significantly reversed MPTP-induced variation of TH-positive neurons and TUNEL-positive percent (figures 2 and 3 and table 1).

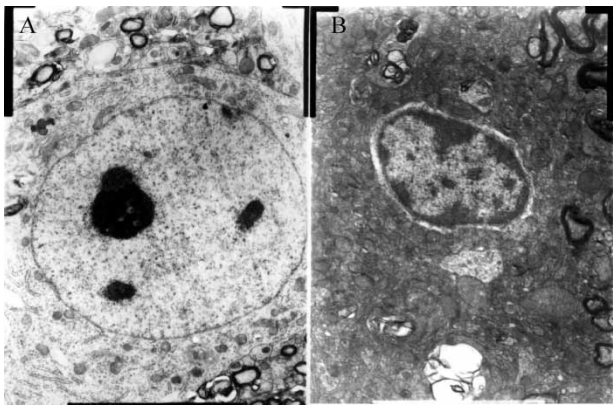

Figure 1. TEM micrographs of mouse SNc. (A, control): typical large neuron nucleus with dispersed chromatin is visible; dopaminergic neurons with apoptotic features, as shown by chromatin condensation, are visible in (B).

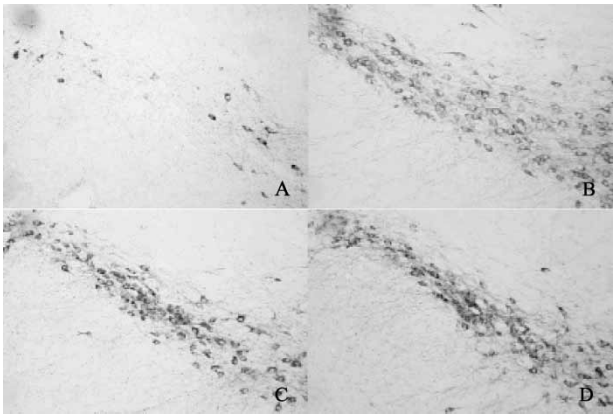

Figure 2. TH immunostaining of mouse SNc neurons. The number of TH-positive neurons decreased in the SNc of MPTP model [MPTP sc 20 mg kg^{-1}, for 8 days (A)], compared with control group (B) and increased in the SNc of pretreatment group [ginsenoside Re ig 26, 13 mg kg^{-1} for 13 days (C, D)], compared with model group. $P < 0.01$ ($\times$ 100).

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2.2 Effects of Re on expression of Bcl-2, bcl-2 mRNA and Bax, bax mRNA

In the MPTP model, a marked decrease of Bcl-2 protein and *bcl-2* mRNA in SNc, as well as a marked increase of Bax and *bax* mRNA was observed, as compared with the control group. Contrarily, after pretreatment with Re (13, 26 mg kg^{-1}), expressions of Bcl-2 protein and *bcl-2* mRNA were increased significantly, and expressions of Bax protein and *bax* mRNA were decreased significantly (figures 4–6).

2.3 Effect of Re on caspase-3 activation

Since caspase-3 protease is synthesized as a 32 kDa inactive precursor, which is proteolytically cleaved to produce the active caspase-3 fragment, namely 17 and 12 kDa subunits, we examined the cleavage of caspase-3 protein in response to apoptosis. As shown in figure 7, a 32 kDa caspase-3 protein band disappeared upon the induction

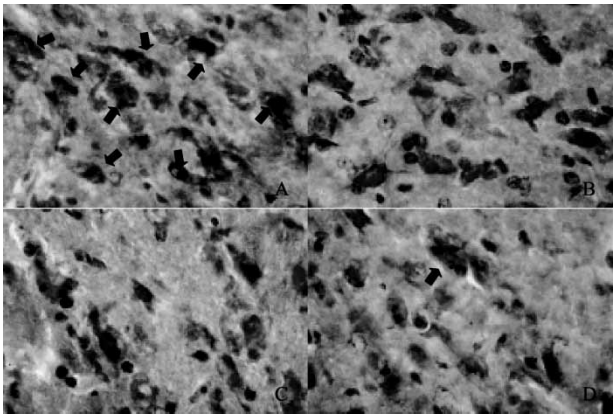

Figure 3. TUNEL staining of mouse SNc neurons. The number of TUNEL-positive neurons increased in the SNc of the MPTP model [MPTP sc 20 mg kg^{-1} for 8 days (A)], compared with control group (B) and decreased in the SNc of pretreatment group [ginsenoside Re ig 26, 13 mg kg^{-1} (C, D)] compared with the model group. $P < 0.01$ ($\times$ 400).

Table 1. TH immunohistochemistry staining and TUNEL staining in the MPTP treated mouse SNc neurons.

Groups	*TH-positive neuron*	*TUNEL-positive ratio (%)*
Control group	11.78 ± 2.86	26.32 ± 6.16
Model group	4.69 ± 0.92[a]	51.50 ± 2.97[a]
Re 6.5 mg kg^{-1}	6.12 ± 2.35	39.50 ± 2.50
Re 13 mg kg^{-1}	9.71 ± 2.99[b]	28.65 ± 2.30[b]
Re 26 mg kg^{-1}	10.59 ± 2.21[b]	26.60 ± 2.77[b]

[a]$P < 0.01$ model *vs.* control group, [b]$P < 0.01$ ginsenoside Re group *vs.* model group.

of apoptosis by the treatment with MPTP, whereas after pretreatment with Re (13, 26 mg kg^{-1}) the 32-kDa band showed a noticeable expression compared with the MPTP model group.

2.4 Effect of Re on expression of iNOS

In the MPTP model group, the amount of iNOS-positive cell in the SNc was increased markedly, while decreased markedly after pretreatment with Re (see figures 8 and 9).

3. Discussion

The pathological hallmarks of Parkinson's disease (PD) are a loss of dopaminergic (DA) neurons in the mesencephalon and the presence of Lewy bodies in altered neurons. In PD, damage to dopaminergic neurons may occur through oxidative stress and/or mitochondrial impairment and culminate in activation in an apoptotic manner. The exact cause of this neuronal loss is still unknown, but recent histological studies performed on brains from Pakinsonian patients suggest that nigral DA neurons die by apoptosis [5–7], and substantial evidence indicates that MPTP-induced neuronal death includes apoptosis. Addition of MPP^+ on cultured mesencephalic neurons, and cerebellar granular cells, produced the characteristic feature of apoptosis in neurons. Accordingly, anti-apoptosis therapy has received much attention.

In our studies, the systemic administration of 20 mg kg^{-1} MPTP for 8 consecutive days on C57BL/6 mice led to the death of dopaminergic neurons in substantial nigral in an apoptotic

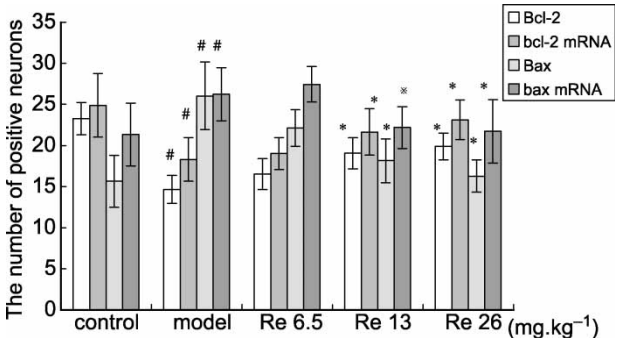

Figure 4. Expression of Bcl-2, *bcl-2* mRNA, Bax, *bax* mRNA in the MPTP treated mouse SNc neurons. #$P < 0.01$ model *vs.* control group. $P < 0.05$, *$P < 0.01$ ginsenoside Re group *vs.* model group.

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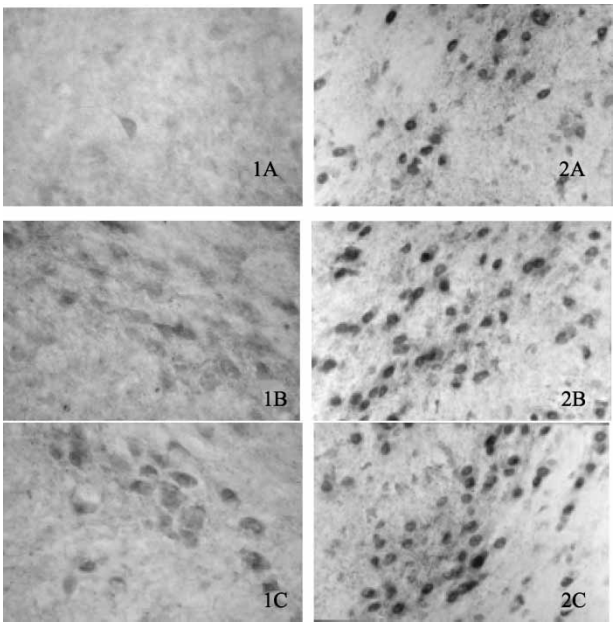

Figure 5. Expression of Bcl-2 protein (1) and *bcl-2* mRNA (2) in the MPTP-treated mouse SNc neurons. (A) model group (MPTP sc 20 mg kg^{-1} for 8 days); (B) control group; (C) pretreatment group (ginsenoside Re ig 26 mg kg^{-1}) (× 400).

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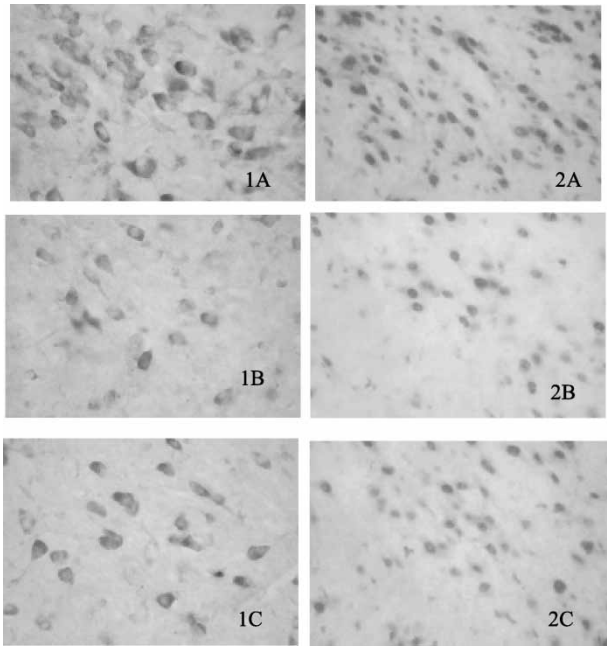

Figure 6. Expression of Bax protein (1) and *bax* mRNA (2) in the MPTP treated mouse SNc neurons. (A) model group (MPTP sc 20 mg kg^{-1} for 8 days); (B) control group; (C) pretreatment group (ginsenoside Re ig 26 mg kg^{-1}) (× 400).

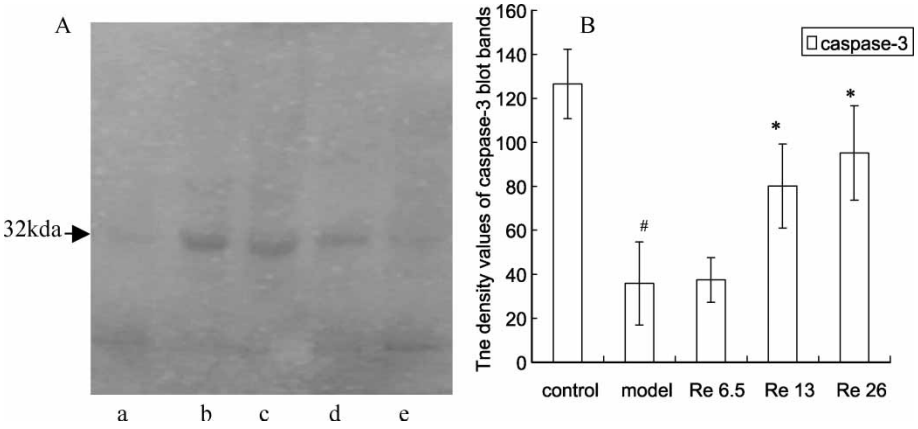

Figure 7. Western blots show the appearance of caspase-3 in the mouse SNc. treated with MPTP. (A) Immunoblotting for activated caspase-3. Lanes: (a) indicates MPTP model group; (b) control group; (c, d and e) indicate Re 26, 13, 6.5 mg kg^{-1} pretreatment groups, respectively. (B) Average density values of caspase-3 blot bands in the MPTP treated mouse SNc neurons. Densitometric analysis revealed that the Re (26, 13 mg kg^{-1}) prevent the activation of caspases-3. $^{\#}P < 0.01$ model *vs.* control group; $^{*}P < 0.01$ ginsenoside Re group *vs.* model group.

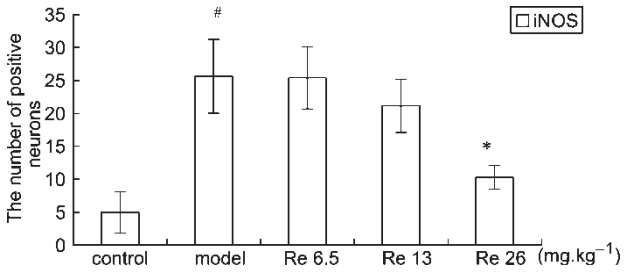

Figure 8. iNOS immunohistochemistry staining in the MPTP treated mouse SNc neurons. $^{\#}P < 0.01$ model *vs.* control group; $^{*}P < 0.01$ ginsenoside Re group *vs.* model group.

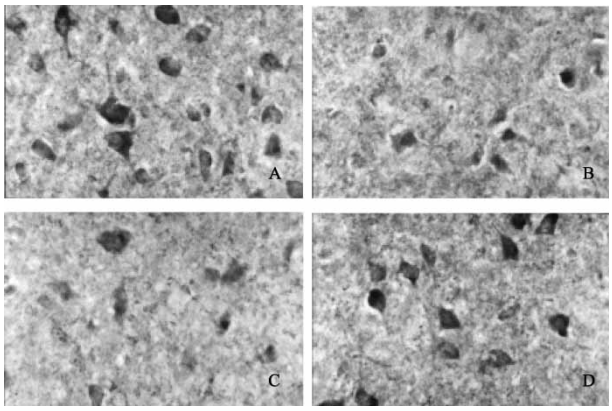

Figure 9. iNOS immunohistochemistry staining in the MPTP treated mouse SNc neurons. (A) model group (MPTP sc 20 mg kg^{-1}for 8 days); (B) control group; (C) pretreatment group (ginsenoside Re ig 26 mg kg^{-1}); (D) pretreatment group (ginsenoside Re ig 13 mg kg^{-1}) (× 400).

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manner, which was confirmed by TEM, TH immunohistochemical staining and TUNEL staining. A decrease of dopaminergic neurons and an increase of apoptotic cell proportion in the SNc were observed in the model group, but pretreatment with Re reduced the above toxicity of MPTP to dopaminergic neurons in the SNc. It is suggested that Re offers potential dosage-dependent protection against MPTP-induced neurotoxicity (Re high dosage group $>$ Re middle dosage group $>$ Re low dosage group). The above pretreatment Re groups significantly differed from the model group, apart from the Re low dosage group ($P < 0.01$).

Bcl-2, a 26 kDa protein, belongs to a family of proteins including members that can act as agonists in cell death, such as Bax and Bak, and others capable that can inhibit, such as Bcl-2 and Bcl-X_L [8]. Bcl-2 might function as an inhibitor of Bax, thus inhibiting cell death. The ratio between the various members of the family could thus be instrumental in deciding between cell survival and cell death. Yang *et al.* [9] indicated that the effects of MPTP on the loss of dopaminergic neurons are thus reduced by overexpression of Bcl-2. Some studies showed that the pro-apoptotic protein Bax is highly expressed in the SNc and that its ablation attenuates SNc developmental neuronal apoptosis. In adult mice, there is an up-regulation of Bax in the SNc after MPTP administration and a decrease in Bcl-2. These changes parallel MPTP-induced dopaminergic neurodegeneration. They also showed that mutant mice lacking Bax are significantly more resistant to MPTP than their wild-type littermates [10]. In our study, the levels of bax mRNA and Bax protein were reduced, and the levels of *bcl-2* mRNA and Bcl-2 protein were enhanced in SNc in Re pretreatment groups, which reversed the levels of these parameters in the MPTP model group. Thus, these results demonstrate that the increased expression of Bcl-2 and *bcl-2* mRNA, as well as decreased expression of Bax and *bax* mRNA, may be critical mechanisms in the protective effects of Re against MPTP-induced apoptosis. Moreover, expressions of Bcl-2 and Bax were consistent with that of their mRNA, showing that the regulation of this anti-apoptotic effect proceeded at the level of gene transcription or translation at least.

The aspartate-specific cysteine proteases (caspases), several of which are known as ICE (interleukin-converting enzymes), form a family with at least 12 members that cleave after aspartate residues [11]. Extensive *in vitro* studies in non-neuronal and neuronal cell systems indicate that caspases are effectors of apoptosis [12]. The caspases can induce cell neuronal death when overexpressed. Conversely, the absence of caspases leads to anomalous neuronal development [13]. In neurons, several lines of evidence indicate that caspase-3 (CPP32/Yama/Apopain), a 32 kDa cytosolic protein, plays a major role in the executive phase of apoptosis. In MPTP and 6-hydroxydopamine (6-OHDA) models of PD, neurotoxins exerted their proapoptotic actions *via* activation of caspase-3-like proteases in neuronal *in vitro* models [14]. Caspase-3 activation is required during the effector phase of cell death and is not a consequence of cell death. It might thus be possible to block this activity and so prevent cell death from progressing. Accordingly, we found that caspase-3 activation was markedly inhibited after pretreatment with Re compared with the MPTP model group, showing that protection of Re on MPTP-induced apoptosis may occur *via* inhibiting cleavage of caspase-3 producing apoptotic activity.

Nitric oxide (NO), formed by the conversion of L-arginine into L-citrulline by NO synthase (NOS), is involved in important physiological functions of the CNS. Depending on the redox state of NO, it can act as a neurotoxin or it can have a neuroprotective action. Data suggest that excess NO may have a role in the pathogenesis of neurodegenerative disorders such

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as Parkinson's disease. Additionally, previous studies indicate that inhibition of two NO-synthesizing enzymes, neuronal NOS (nNOS) and inducible NOS (iNOS), displays neuroprotective effects in the MPTP model of PD [15]. The present study showed that expression of iNOS was reduced after pretreatment with Re in comparison with the MPTP model, suggesting the protective role of Re on MPTP-induced apoptosis may be associated with reducing NO production by decreasing iNOS expression.

We have reached the following conclusions: (1) Administrating 20 mg kg^{-1} MPTP at 24 h intervals for 8 consecutive days can induce death of dopaminergic neurons in an apoptotic manner. (2) Re can protect dopaminergic neurons in SN from MPTP-induced apoptosis through increasing the level of Bcl-2 protein and *bcl-2* mRNA expression, decreasing the level of Bax protein and *bax* mRNA expression, as well as inhibiting the activation of caspase-3.

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4. Experimental

4.1 Establishment of animal model and grouping

Experiments were conducted on 8–9-week-old C57BL/6 mice, body weight 20–25 g, maintained, under standard animal care conditions, on a 12 h day–night cycle and given food and water *ad libitum*. All studies were carried out in accordance with the protocol of the local animal care and use committee. Animals were divided into five groups ($n = 8$): The model group per time period received an i.s.c. injection of MPTP 20 mg kg^{-1} day^{-1} at 24 h intervals for 8 consecutive days, while the control group in same paradigms were treated with a equal volume of saline, and the pretreatment groups were pretreated with Re (6.5, 13, 26 mg kg^{-1} day^{-1}, i.g.) respectively for 5 days before MPTP injection and then in the following 8 days these groups with the same dosage were treated with Re 2 h before receiving an MPTP injection as mentioned.

4.2 Tissue preparation

All animals were killed by decapitation 24 h after the last injection. For each mouse, one of the two substantia nigra was dissected, immediately frozen on dry ice, and stored at 80°C for Western blotting assay. The other hemi-mesencephalon was placed in chilled 4% paraformaldehyde in phosphate buffer (PB, 0.1 mmol L^{-1}, pH 7.4), fixed at 4°C for 24 h, and then cryoprotected in 20% glycerol at 4°C for TUNEL staining and immunohistochemistry assay.

4.3 Transmission electron microscopy (TEM)

Fragments from SNc were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 3 h at 4°C and washed with the same buffer containing 7% sucrose; the specimens were then post-fixed in 1% OsO_4 for 2 h at 4°C and washed repeatedly in buffer. Thereafter, specimens were dehydrated in acetone, passed through propylene oxide, and finally embedded in epoxy resin. Ultrathin sections (60 nm) were collected on copper grids

and stained with uranyl acetate and lead citrate. Specimens were observed with a Zeiss EM109 at 100 kV.

4.4 Immunohistochemistry of tyrosine hydroxylase (TH), Bcl-2, Bax and iNOS

The nigra was serially sectioned, and the section was incubated with 1% bovine serum albumin (BSA)/3% H_2O_2 in phosphate-buffered saline (PBS, 0.01 mmol L^{-1}, pH 7.4) for 1 h at room temperature, and then incubated with the primary antibodies, which were rabbit anti-mouse polyclonal antibodies, including TH (1:1000, Sigma, USA), Bcl-2 (1:100, Santa Cruz, USA), Bax (1:200, Santa Cruz, USA) and iNOS (1:50, Santa Cruz, USA), overnight at 4°C. Sections were washed with PBS, then incubated in sequence with biotinylated anti-rabbit IgG and SABC-reagent (1:400, Vectastain ABC Kit, Vector, USA) for 1 h at room temperature, and finally rinsed with diaminobenzidine (DAB) to produce a brown-yellow precipitate in the plasma of the positive cell. After dehydration, transparence and obstructing glass piece, a series of stained sections were analysed under light microscopy.

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4.5 Detection of apoptotic cells by TUNEL

Nigral sections were incubated with 0.1% Triton X-100 in Tris–HCl buffer saline (TBS) for 1 h at room temperature, and then incubated with 3% H_2O_2; 10 min later, sections were washed with distilled water for 2 min (3 ×). Sections were then incubated, in order, with TdT reaction mixture, biotinylized digoxin Ab and SABC reagent (TUNEL detection kit, Boster, China) for 2 h at 37°C. Finally, sections were visualized with DAB to produce a dark brown precipitate. Stained sections were analysed under light microscopy.

4.6 Detection of caspase-3 by Western blotting analysis

Samples were boiled for 5 min, and equal amounts of protein were separated by 12.5% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked in 3% bovine serum albumin in Tris–HCl buffer saline for 1 h at room temperature and then incubated with primary Ab, caspase-3 (CPP32/Yama/apopain, 1:50 dilution in Tris–HCl buffer saline, pH 7.6) overnight at 4°C, and subsequently incubated, in order, with secondary Ab and SABC reagent for 2 h at room temperature. Finally, the membranes were gently rinsed blot in DAB solution until a brown precipitate formed. Molecular sizes were determined by the relative mobilities of prestained molecular weight markers. Densitometric analysis was performed with a computer using a gel image analysis program.

4.7 Detection of Bcl-2 and bax gene expression by in situ hybridization histochemistry (ISHH)

Sections were saturated with 0.3% H_2O_2–methanol solution for 30 min at room temperature and then washed with distilled water. After uncovering the mRNA nucleic acid segment with pepsin, diluted in 3% citric acid, for 3 min at room temperature, the sections were incubated with pre-hybridization solution for 3 h at 42°C; the digoxin-labeled probe was then added onto the sections for hybridization overnight at 42°C. Sections were then washed, in order, with 2 × SSC solution for 5 min (2 ×), 0.5 × SSC solution for 10 min (2 ×), 0.2 × SSC

solution for 15 min (2 ×) at 37°C. The addition of a blocking solution at 37°C for 30 min, then a mice antibody against digoxin for 1 h at 25°C, followed by washing with 0.02 mmol L^{-1} PBS solution were performed in sections. Thereafter, SABA reagent was added onto the sections, and 1 h later the sections were washed with 0.02 mmol L^{-1} PBS solution at room temperature. Finally, after showing color with DAB, dehydration, transparence and obstructing glass piece were performed. A series of stained sections were analysed under light microscopy.

4.8 Statistical analysis

Data are expressed as mean ± SD. The TUNEL-positive percent was calculated as: (TUNEL-positive cells/total cells) × 100%. Bands of Western blotting were calculated by average densitometric analysis. The statistical significance of differences between groups was determined *via* one-way analysis of variance (ANOVA). Statistical significance was assumed at $P < 0.05$.

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