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Asiaticoside: Attenuation of neurotoxicity induced by MPTP in a rat model of Parkinsonism via maintaining redox balance and up-regulating the ratio of Bcl-2/Bax

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ABSTRACT

In this study, we investigated the neuroprotective effects of asiaticoside, a triterpenoid saponin isolated from the Chinese medicinal herb *Centella asiatica*, in the rats model of Parkinsonism induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Rats were first injected with MPTP. One day after surgery, asiaticoside was administered and the behavioral tests were assessed. On 14th day, the rats were sacrificed, substantia nigra (SN) and striatum were dissected, and then dopamine (DA) and its metabolites in striatum and malonyldialdehyde (MDA) contents, reduced glutathione (GSH) level and gene expression level in SN were estimated. Treatment with asiaticoside was found to protect dopaminergic neuron by antagonizing MPTP induced neurotoxicity and to improve locomotor dysfunction. Asiaticoside significantly attenuated the MPTP-induced reduction of dopamine in the striatum. The content of MDA was significantly decreased while the GSH level was significantly increased in asiaticoside-treated groups. In addition, asiaticoside increased the Bcl-2/Bax ratio. These results indicated that asiaticoside was effective in reversing MPTP induced Parkinsonism via its neuroprotective effects including antioxidant activity, maintaining the metabolic balance of DA, and increasing ratio of Bcl-2/Bax.

1. Introduction

Parkinson's disease (PD) is a chronically progressive, age-related neurodegenerative movement dysfunction characterized by progressive resting tremor, rigidity, bradykinesia, postural instability along with non-motoric symptoms like autonomic, cognitive and psychiatric problems (Mandemakers et al., 2007). The neuropathological hallmarks are characterized by massive loss of nigrostriatal dopaminergic neurons in the substantia nigra (SN) pars compacta and the resultant deficiency in the neurotransmitter dopamine (DA) at the nerve terminals in the striatum (Nagatsu and Sawada, 2005). Several hypotheses have been proposed for explaining the progressive and selective neurodegeneration in PD. Mitochondrial dysfunction, which is one of most important hypotheses, has been linked with PD for a long time. The dysfunction of mitochondria, including deficiencies in energy supply, free radical generation, Ca^{2+} buffering, not only involve damage to the organelle and loss of bioenergetic function but also disruption of mitochondrial-dependent redox signaling pathways, and eventually lead to cell death (Gutierrez et al., 2006).

The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) elicited Parkinsonism was first observed in a group of heroin users presented with symptoms similar to PD (Langston et al., 1983). Following research found that the biochemical and cellular changes that occur after administration of MPTP in animals were remarkably similar to those seen in idiopathic PD (Ferro et al., 2005). DA supplementation therapy by L-dopa for PD was established around 1970. However, many patients develop motor complications, and L-dopa-induced dyskinesia is common and difficult to treat. So a much more effective compound that have less side effects is needed to be found out in order to take place of traditional DA supplementation therapy by L-dopa.

Asiaticoside (Fig. 1), a triterpenoid saponin, is isolated from *Centella asiatica* which has a long history of use in India as a memory enhancing drug in ayurvedic literature and has been proved to have some pharmacological activities in central nervous system (Dhanasekaran et al., 2009; Flora and Gupta, 2007; Haleagrahara and Ponnusamy, 2010). Asiaticoside also offers protection against chemical-induced inflammation and hepatotoxicity (Yun et al., 2008; Zhang et al., 2010). Moreover, recent studies indicated that

Abbreviations: PD, Parkinson's disease; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; SN, substantia nigra; DA, dopamine; MDA, malonyldialdehyde; GSH, reduced glutathione; qRT-PCR, quantitative real-time polymerase chain reaction; DOPAC, 3, 4-dihydroxyphenylacetic acid; HVA, homovanillic acid; DHBA, 3, 4-dihydroxybenzylamine; HPLC, high-performance liquid chromatography; LPO, lipid peroxidation; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances.

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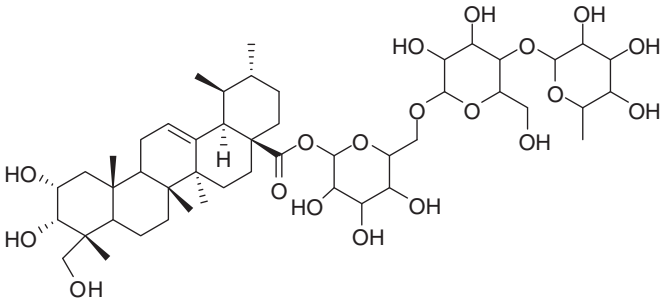

Fig. 1. The structure of asiaticoside.

asiaticoside has shown to rescue B103 rat neuroblastoma cells against $A\beta_{25-35}$ and H_2O_2-induced neurotoxicity (Mook-Jung et al., 1999). MPTP rats have been used as a model of Parkinsonism for evaluating the anti-PD effect of asiaticoside. The effects of asiaticoside on locomotor activity, DA content, malondialdehyde (MDA) concentrations, and reduced glutathione (GSH) levels in nigrostriatal system of MPTP-induced Parkinsonism phenotype in rats were investigated. Additionally, the expressions of Bcl-2 and Bax were also evaluated for elucidating the underlying molecular mechanisms of neuroprotection of asiaticoside.

2. Materials and methods

2.1. Apparatus and reagents

The high-performance liquid chromatography (HPLC) system with electrochemical detection consisted of a solvent delivery module, an ESA sampler, an ODS reverse-phase column (C18 250 mm × 4.6 mm) and an ESA-Coulochem III electrochemical detector with a 5020 guard cell and a 5010 analytical cell. DA, 3, 4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and internal standard 3, 4-dihydroxybenzylamine (DHBA) were purchased from Sigma (USA). All solutions were prepared with ultra pure water (18.2 MΩ-cm) from a Purelab ultra System (ELGA Purelab, UK).

Asiaticoside was kindly provided by Dr. Qizhi Wang (Jiangsu Institute of Botany, Chinese Academy of Science) and the purity of this compound was greater than 97% tested by HPLC analysis.

2.2. Animals

Adult male Wistar rats (170–190 g, Shanghai laboratory Animal Center, Chinese Academy of Science) were used in this study. The animals were fed in a controlled environment (23 ± 1 °C, 12-h light–dark cycle light) with *ad libitum* access to food and water. The animals were cared complied with the Provisions and General Recommendation of the Chinese Experimental Animals Administration Legislation and were approved by the Science and Technology Department of Jiangsu Province.

2.3. Experimental design

Rats were distributed randomly into 6 groups with 6 in each: control group, sham-operated control group, the MPTP (MPTP-lesioned group) group, asiaticoside treated groups (15, 30, or 45 mg/kg/day). All animals underwent stereotaxic surgery and bilateral infusion of MPTP or saline on day 0 into the SN. One day after surgery, the rats received daily intragastric administration (i.a.) of asiaticoside or saline in a volume of 1 ml/kg at 13:00 h for 14 days. Open-field test was conducted on the 1st, 7th and 14th days at 14:00–18:00 h. Ladder-walking was conducted on the 7th and 14th days at 14:00–18:00 h. At the end of these experiments, the rats were sacrificed and dissection of the striatum and SN for other experiments.

2.4. Stereotaxic surgery

The method of brain surgery has been reported previously (Ferro et al., 2005). In brief, the rats were anesthetized by intraperitoneal (i.p.) injection of chloral hydrate at a dose of 400 mg/kg, and then stereotaxically injected bilaterally into the SN with MPTP (Sigma, USA; 1 μmol in 2 μl of saline, 0.35 μl/min) through a 30-gauge needle according to the following coordinates: AP: −5.0 mm, ML: ± 2.1 mm, DV: −7.7 mm from the bregma, midline, and skull surface, respectively. Controls were subjected to the same procedure, but were infused with 2 μl of saline instead of MPTP. Immediately after surgery, the rats were injected with penicillin-G procaine (0.2 ml, 20,000 IU, IM) and the rats were left in a temperature-controlled chamber until they recovered from anesthesia, then they were returned to their home cages.

2.5. Behavioral test

2.5.1. Open-field test

Open-field consisted of square arena (120 × 120 cm). The square arena was divided into 16 sub squares. The rats were submitted to the open-field on the 1st, 7th and 14th days after the surgery. The test placed the rat in the center of the arena. The behaviors of the rats were then observed for 5 min. After each test, the apparatuses were cleaned. The number of crossings (The rats cross the sub squares boundaries with paws), peripheral ambulation time (The rats crawl time with their paws), and immobilization time were scored by an observer blind to the treatment received by each rat.

2.5.2. Ladder walking

Crossing the 'horizontal ladder' required the rats accurately place their limbs on the bars. The test was estimated coordinating ability of rats' hindlimb and forelimb. Rats were trained to walk across the ladder rung and the rungs were irregularly spaced out (1–3 cm apart). The rung space was not altered during all experimental days. Rats were trained to walk across the ladder for 3 days before the test and the average performance of three times in days 7 and 14 was recorded.

2.6. HPLC analysis DA and its metabolites

On the 14th day, the rats were decapitated and the brains were quickly removed within 30 s and immediately dropped into ice-cold saline. The brains were dissected over ice. Each group we randomly choose three rats whose striatum from the left hemisphere were weighted and homogenized at perchloric acid (0.1 M) in a glass homogenizer. The levels of DA and its metabolites were detected by HPLC analysis previously described by our laboratory (Li et al., 2011). The results were calculated and expressed as ng/mg tissue weight.

2.7. Assay of substantia nigra MDA level

The lipid peroxidation (LPO) of SN was studied by measuring the malonyldialdehyde (MDA) level by a colorimetric method involving thiobarbituric acid (TBA) adduct formation. Homogenate of SN was prepared, and the amounts of TBA reactive substances (TBARS) such as MDA were measured by the reaction with TBA using a commercial TBARS Assay Kit (Cayman Chemical Co.). Operation followed the manufacturer's protocol. The MDA were determined by comparison with standards and normalized to protein content.

2.8. Assay of SN GSH concentration

The effect of asiaticoside treatment on GSH level was evaluated using a commercial kit (Cayman Chemical Co.). Homogenate of SN was used for GSH measurements using GSH Assay Kit (Cayman Chemical) following the manufacturer's protocol. The GSH determined by comparison with standards and normalized to protein content.

2.9. RNA extraction and real-time PCR analysis

After the treatment for 14 days, the rats were decapitated rapidly and their brains were dissected on ice. Each group we randomly choose three rats whose SN from the left hemisphere were homogenized in 1 ml of TRIzol (GIBCO BCR Inc., Shanghai, China) using a glass homogenizer. The total RNA was isolated according to the manufacturer's protocol. The primers for PCR were used as follows: Bcl-2: forward 5′-ATCTTCTCCTTCCAGCCTGA-3′ and reverse 5′-TGCAGCTGACTGGACATCTC-3′; Bax: forward 5′-CTGCAGAGGATGATTGCTGA-3′ and reverse 5′-GAGGAAGTCCAGTGTCCAGC-3′ (Zhou and Zhu, 2000); β-actin: forward 5′-AGC CAT GTA CGT AGC CAT CC-3′ and reverse 5′-CTC TCA GCT GTG GTG GTG AA-3′ (Nanashima et al., 2008). The SYBR green PCR Master Mix was used for real-time PCR analysis. The cycle time values of the interested genes were first normalized with β-actin of the same sample, and then the relative difference between control and each treatment group was calculated and expressed as a relative reduction, setting the control group at 100%.

2.10. Protein assay

The protein content of the supernatant was measured by the Bradford method, using bovine serum albumin (Sigma) as the standard (Bradford, 1976).

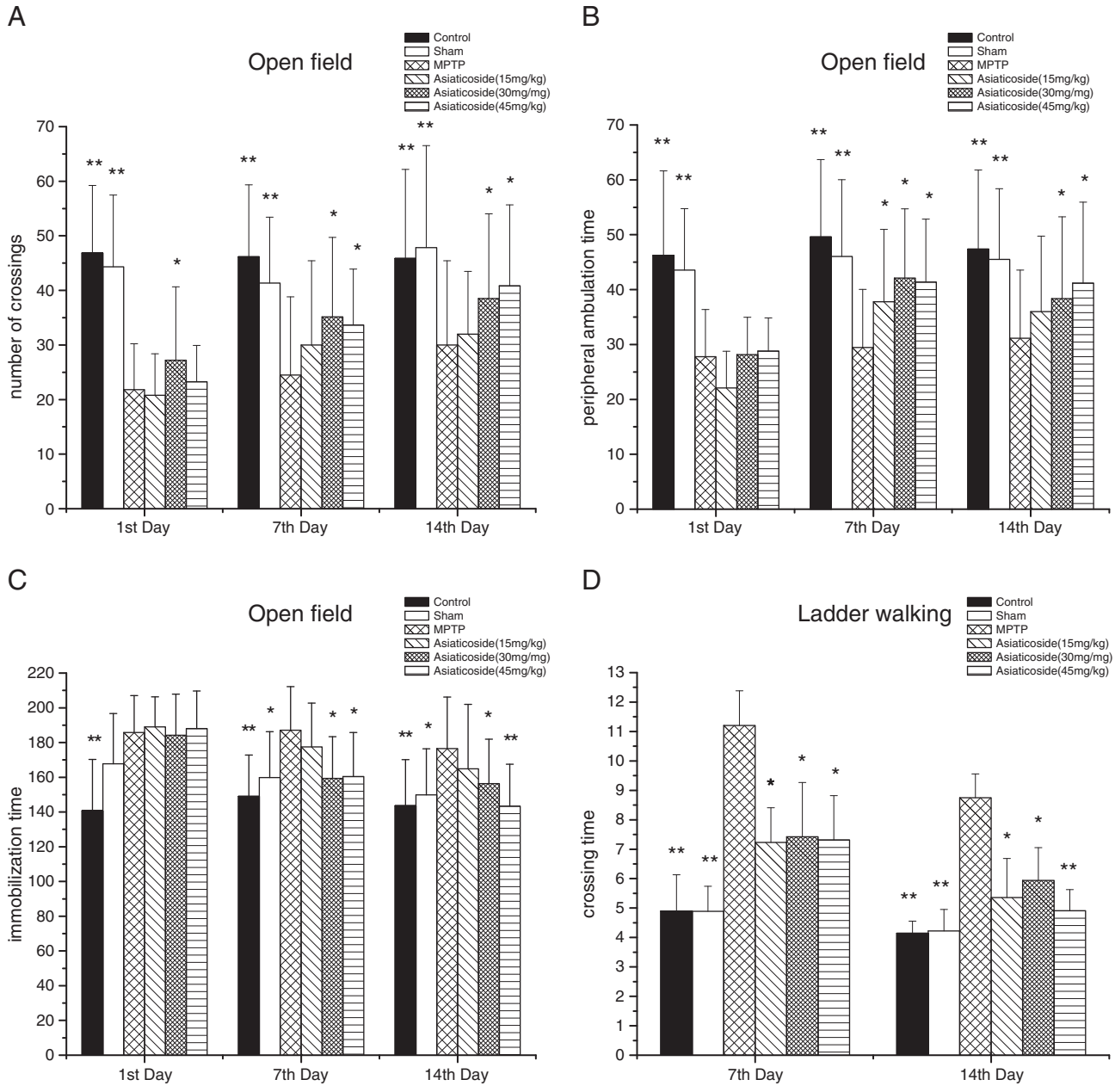

Fig. 2. Asiaticoside was observed to improve MPTP-induced motor deficits in a rat model of Parkinsonism. A, number of crossings in open field test; B, peripheral ambulation time in open field test; C, immobilization time in open field test; D, crossing time in ladder walking test. *P<0.05, **P<0.01 *versus* the MPTP group.

2.11. Statistical analysis

The results are expressed as means ± SD and analyzed by one-way analysis of variance (ANOVA) Duncan's multiple range test was applied post-hoc to determine differences that were considered statistically significant at $P<0.05$.

3. Results

3.1. Effect of asiaticoside on MPTP-induced motor behavior in rats

3.1.1. Open-field test

Fig. 2 showed the effects of different doses of asiaticoside on number of crossings (Fig. 2A), peripheral ambulation time (Fig. 2B) and immobilization time (Fig. 2C). Significant effects were produced by MPTP, which suppressed number of crossings [$F_{1st}(5,30)=7.330$, $P<0.01$; $F_{7th}(5,30)=3.415$, $P<0.05$; $F_{14th}(5,30)=3.084$, $P<0.05$], peripheral ambulation time [$F_{1st}(5,30)=6.858$, $P<0.01$; $F_{7th}(5,30)=3.481$, $P<0.05$; $F_{14th}(5,30)=3.241$, $P<0.05$] and increased immobilization time [$F_{1st}(5,30)=3.689$, $P<0.05$; $F_{7th}(5,30)=3.878$, $P<0.01$; $F_{14th}(5,30)=3.348$, $P<0.05$] in all three times of open field tests. This suppression of number of crossings, peripheral ambulation time and increase of immobilization time were attenuated on the 7th and 14th days of treatment with asiaticoside (Fig. 2A–C, respectively). However on the 1st day, there was no significant difference in number of crossings, peripheral ambulation time and immobilization time.

3.1.2. Ladder walking

Deficits in limb coordination and limb placing were examined by assessing the rat's ability to navigate across a runway with irregularly spaced rungs. One-way ANOVA showed that the MPTP group markedly spent longer time in crossing the runway than sham operated and control groups [$F_{7th\ day}(5,30)=18.076$, $P<0.01$; $F_{14th\ day}(5,30)=21.610$, $P<0.01$]. This result revealed that MPTP group had significant movement coordination impairment. Asiaticoside treated groups (15, 30, 45 mg/kg) decreased running time in comparison with the MPTP group (Fig. 2D).

3.2. Levels of DA and its metabolites

Bilateral MPTP injections in SN caused a reduction of DA, DOPAC and HVA. [$F_{DA}(5,12)=8.832$, $P<0.01$; $F_{DOPAC}(5,12)=3.371$, $P<0.05$; $F_{HVA}(5,12)=3.748$, $P<0.05$]. Asiaticoside attenuated the reduction in striatum induced by MPTP. As shown in Fig. 3, the contents of DA in asiaticoside treated groups (15, 30, 45 mg/kg) were higher than the MPTP group and the contents of DOPAC and HVA were only significantly elevated in high-dose asiaticoside treated group.

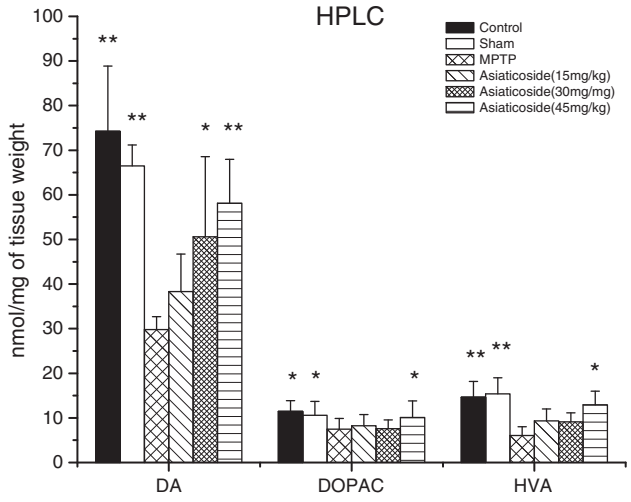

Fig. 3. Treatment with asiaticoside significantly and dose-dependently attenuated MPTP-induced dopamine depletion in MPTP-induced Parkinsonism in rats. *P<0.05, **P<0.01 *versus* the MPTP group.

3.3. Effect of asiaticoside on MPTP-induced MDA level in rats

The most abundant carbonyl product of LPO was taken as an index of oxidative stress. TBARS are a marker of LPO which is indicative of MDA formation and lipid damage and is a well-established method for screening and monitoring LPO. In this study, there was a significant increase in MDA in SN of MPTP group compared to the control group and sham group [$F(5,12)=53.222$, $P<0.01$]. Treatment with different dose of asiaticoside (15, 30, 45 mg/kg) for 2 weeks on the MPTP-induced Parkinsonism in rats resulted in a significant reduction in MDA levels compared to MPTP group (Fig. 4A).

3.4. Effect of asiaticoside on MPTP-induced GSH concentration in rats

In order to investigate whether the neuroprotective effect of asiaticoside was caused by antioxidant actions, we measured GSH

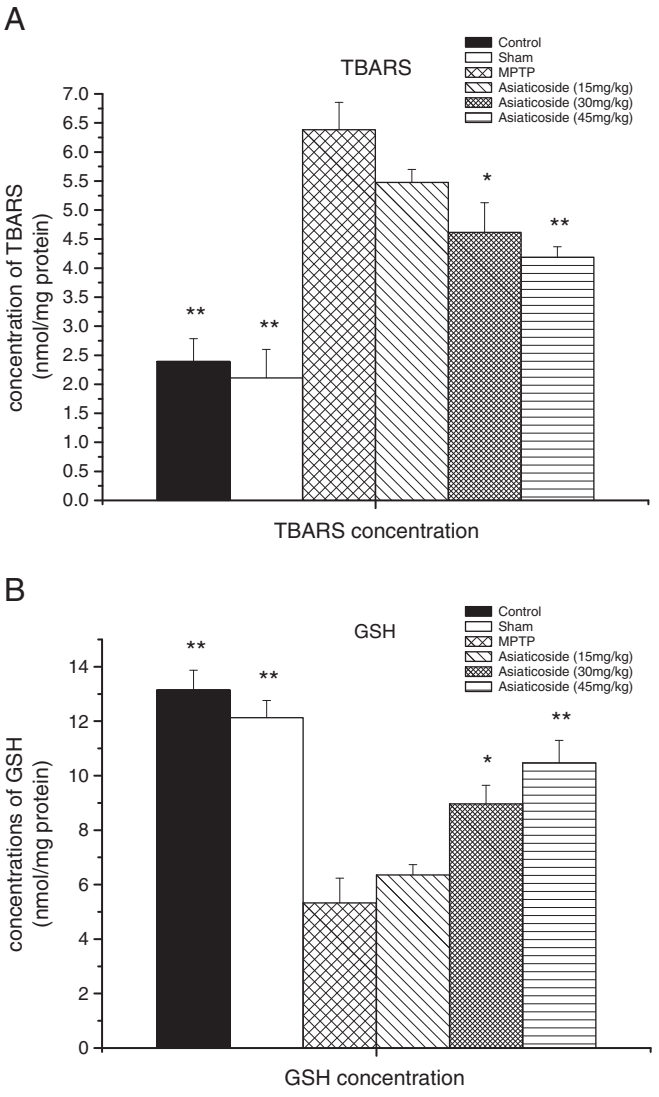

Fig. 4. Asiaticoside maintained the balance of redox by decreasing the levels of MDA and increasing the content of GSH in SN. A, The effects of asiaticoside on the levels of MDA as measured by TBARS generation in SN. B, The effects of asiaticoside on MPTP-induced GSH depletion in SN. *P<0.05, **P<0.01 *versus* the MPTP group.

concentration as an indicator of cellular redox status in the SN. After MPTP treatment, the levels of GSH were significantly depleted [$F(5,12)=57.7$, $P<0.01$] as shown in Fig. 4B. Asiaticoside administration (30, 45 mg/kg) significantly restored GSH concentration compared with the MPTP group.

3.5. Effect of asiaticoside on MPTP-induced gene expression in rats

The expression of apoptosis-related genes Bcl-2 and Bax were changed by MPTP in SN. As shown in Fig. 5, compared with the control group and sham group, the mRNA levels of Bcl-2 and Bax in the MPTP group were significantly decreased and increased, respectively [$F_{bcl\text{-}2}(5,12)=77.728$, $P<0.01$; $F_{bax}(5,12)=23.680$, $P<0.01$; $F_{bcl\text{-}2/bax}(5,12)=90.374$, $P<0.01$]. In asiaticoside treated groups (15, 30, 45 mg/kg), the mRNA levels of Bcl-2 and Bax were significantly increased and decreased in a dose-dependent manner compared to the MPTP group, respectively. Also, we calculated the Bcl-2/Bax ratio and found that it was significantly elevated in the asiaticoside treated groups in comparison with the MPTP group.

4. Discussion

Locomotor dysfunction is a kind of clinical symptoms of PD. The central motor manifestations are linked to death of dopaminergic neurons projecting from the SN to the striatum. Previous researches demonstrated that bilaterally SN MPTP-lesioned rats could well mimic motor deficit (Ferro et al., 2005). This model can also cause DA depletion in striatum which is another pathologic feature of Parkinsonism. In this study, we examined the effect of asiaticoside on the motor function and striatal DA levels. Treatment of asiaticoside not only improved movement dysfunction induced by MPTP in the open field tests and ladder walking test, but also prevented the reduction of DA and DOPAC contents in the striatum. These findings indicated that asiaticoside could reverse MPTP induced motor deficits and magnitude of depleted levels of DA in rats.

Oxidative mechanism in the pathogenesis of PD has been proved by the knowledge that aging is the most important risk factor for developing PD (Liang et al., 2007). Proteins, lipids damaged by oxidative stress have participated in PD pathological process (Dexter et al., 1989; Alam et al., 1997). On the other side, LPO is a well-known mechanism of cellular injury initiated by ROS (Sayre et al., 2001). The MDA is the most cytotoxic aldehydes produced in the process of LPO and MDA production could reflect oxidative damage to lipids (Esterbauer et al., 1991). GSH is the most important thiol containing antioxidant in the brain (Meister and Anderson, 1983) and it plays a pivotal role in preventing oxidative damage. GSH also have been used as a biomarker of oxidative stress in biological systems (Reed and Savage, 1995). Depletion of GSH has been observed in the SN of PD patients and in the MPTP model (Ferraro et al., 1986). In our studies, asiaticoside could decrease the concentration of MDA and increase the concentration of GSH. The recovering balance of redox in tissue microenvironment is the most likely mechanisms by which asiaticoside exerted neuroprotection effects.

The Bcl-2 family members reside upstream of irreversible cellular death and focus much of their efforts at the level of mitochondria, and they play a pivotal role in deciding cell fate (Gross et al., 1999). To search for the possible mechanism for the neurotrophic action of asiaticoside, we detected the expression of Bcl-2 and Bax genes with real-time PCR. Bcl-2, which is a member of Bcl-2 family, functions as an antagonist of a central mechanism operative in cell death (Hockenbery et al., 1993). Bcl-2 has the neuroprotective effect against MPTP induced depletions of the DA metabolites DOPAC and HVA (Yang et al., 1998). Bcl-2 also inhibits neuron death through decreasing generation of ROS (Kane et al., 1993) and enhancing the patience to oxidative stress (Mirkovic et al., 1997). Bcl-2 can increase the amount of GSH which is a unique antioxidant in which it possesses intrinsic antioxidant activity as well as serving as a cofactor for a number of cellular detoxification pathways (Liang et al., 2007). Bax, which is a pro-apoptotic molecule, becomes an integral membrane protein and cross-linkable as a homodimer following a death stimulus and blocks the anti-apoptotic effect of Bcl-2 (Gross et al., 1999). More recently, it was shown that the ratio of Bcl-2/Bax in the cell determines to a large extent whether the cell initiates apoptosis or not (Del-Poeta et al., 2003). This study demonstrates that asiaticoside can increase the expression of Bcl-2, decrease the expression of Bax (hence, a higher Bcl-2/Bax ratio), and increase the resistance of SN to the damage caused by the MPTP induced Parkinsonism in rats.

In conclusion, our results demonstrated that asiaticoside, a triterpenoid saponin isolated from *Centella asiatica*, had a potent neuroprotective effect on the MPTP rats model of Parkinsonism. The neuroprotective effect of asiaticoside may be mediated through maintaining redox balance and up-regulating the ratio of Bcl-2/Bax. This is an early stage of study of neuroprotective effect of asiaticoside and the detailed mechanisms of action need further clarification.

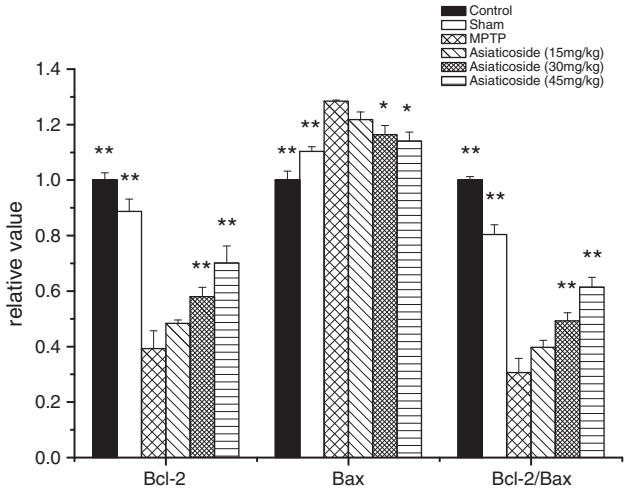

Fig. 5. Asiaticoside not only down-regulated the expression of Bax but also blocked the reduction of Bcl-2 in SN induced by MPTP assessed by real-time PCR. Asiaticoside could significantly increase the ratio of Bcl-2/Bax in SN, *P<0.05, **P<0.01 *versus* the MPTP group.

Conflict of interest

There are no conflicts of interest.

Acknowledgments

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