Neuroprotective Effect of Kaempferol against a 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine-Induced Mouse Model of Parkinson’s Disease

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Preliminary studies in our laboratory have shown that kaempferol derivatives prevent oxidative stress-induced cell death in a DJ-1-dependent manner \textit{in vitro}, and that DJ-1 was a causative gene product of a familial form of PD\textsuperscript{17}, indicating that kaempferol might be neuroprotective in PD. However there has been no systematic research on this topic \textit{in vivo} so far. The purpose of the present study was to evaluate the potential neuroprotective effects of kaempferol in the mouse model of MPTP-induced dopaminergic neuronal damage, and explore its possible mechanism.

**MATERIALS AND METHODS**

**Materials** MPTP and kaempferol (Fig. 1) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Assay kits for SOD, GSH-PX, MDA and MAO-B were purchased from Jiancheng Bioengineering Institute (Nanjing, China). Goat polyclonal antibody against tyrosine hydroxylase (TH) antibody showed that medication of kaempferol could prevent the loss of TH-positive neurons induced by MPTP. Taken together, we propose that kaempferol has shown anti-parkinsonian properties in our studies. More work is needed to explore detailed mechanisms of action.

Key words kaempferol; 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; neuroprotection; oxidative stress; Parkinson’s disease
was purchased from Chemicon International (Temecula, CA, U.S.A.). Histostain™-SP and dianminobenzidine (DAB) kits were purchased from Zhongshan Goldenbridge Biotechnology (Beijing, China).

Animal Grouping and Treatment  Adult male C57BL/6 mice with an initial experimental weight of 20—25 g (8 weeks old) were purchased from the Laboratory Animal Center of Peking University Health Science Center (Beijing, China). These mice met the approval of the local animal committee with confirmation number SCXK (Jing) 2007—0001. Animals were housed in a controlled environment (22±2 °C) with food and water available ad libitum. All experiments were performed under the Guidelines of the Experimental Laboratory Animal Committee of Peking University Health Science Center and were in strict accordance with the principles and guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Animals were acclimatized for 1 week prior to experimentation, then randomly divided into the following six groups (12 mice per group): group A (vehicle control group, an equal volume of normal saline); group B (MPTP model group, pre-treatment with an equal volume of saline); groups C, D and E (low, moderate and high doses of kaempferol, pre-treatment with kaempferol at doses of 25, 50 and 100 mg/kg, respectively); group F (positive-control group, pre-treatment with selegiline at dose of 15 mg/kg). All groups were administered the respective pre-treatment compounds orally (per os (p.o.)) every 24 h for 14 consecutive days, with the first day of administration designated as day 1. The PD mouse models for groups B, C, D, E, F were generated by five consecutive injections of MPTP at a dose of 18 mg/kg every 24 h from day 10 to day 14. The preparative motor activity was measured for 5 min.

Behavior Test. Rotarod Performance Test  The mice took part in the rotarod performance test to evaluate motor coordination 1 d after MPTP treatment.19—21 A rotarod machine (Experimental Factory of Peking University Health Science Center, Beijing, P. R. China) with a rotating spindle (diameter 7.3 cm) and five individual compartments was used to test five mice at a time. The surface of the rotating bar was designed to prevent mice gripping onto the surface. In the formal test, the rotation speed was set to 25 rpm. The time that the mice remained on the rotating bar was recorded for three tests for each mouse at 5-min intervals. Data are presented as mean time on the rotating bar over the three tests.

Spontaneous Motor Activity Test  Spontaneous motor activity was assessed with an infrared motion activity system (Experimental Factory of Chinese Academy of Medical Sciences, Beijing, China) that consisted of four Plexiglas cages (23 cm×30 cm, diameter×height) 2 d after MPTP treatment. Each cage was equipped with three infrared beams that continuously detected all vertical and horizontal movements performed by the mouse. The activity was assessed by counting the number of infrared beam crossing the photocell apparatus per 5 min by an automated counting system.22 The spontaneous motor activity was measured for 5 min.

High Pressure Liquid Chromatography (HPLC) Assay for Analysis of Dopamine and Its Metabolite  Brain Tissue Preparation: Six mice from each group were sacrificed by cervical dislocation 3 d after MPTP treatment. The brains were rapidly removed and dissected on an ice-cold plate. For each mouse, the striata from the left and right hemispheres were together transferred into a 1.5 ml plastic vial, weighed and homogenized in iced-cold HClO₄ (0.4 m) using an ultrasonicator. After storage for 1 h in ice, the homogenates were centrifuged at 12000 g for 15 min at 4 °C. The supernatant was then incubated with a mixed buffer (20 mm sodium citrate, 300 mm K₂HPO₄, 2 mm sodium ethylenediamine-tetraacetic acid [Na₂EDTA]) at the ratio (v/v) of 1 : 2 for 1 h in ice and centrifuged at 12000 g for 15 min at 4 °C. The supernatant was collected and filtered through a 0.22-μm filter and subsequently analyzed by HPLC.23

HPLC Assay: The levels of dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) were determined by HPLC, which was equipped with an electrochemical (EC) detector used for quantification.24 Briefly, the striatum was homogenized in 0.1 mol/l HClO₄ containing 0.1 mm EDTA. The levels of dopamine and DOPAC were determined by reference to standard curves. Results were calculated and expressed as mg/g tissue weight.

Tyrosine Hydroxylase (TH) Immunohistochemistry  Four days after MPTP treatment, mice were anesthetized deeply by intraperitoneal (i.p.) injection of 10% chloral hydrate and perfused through the left ventricle with normal saline, followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4. Brains were removed and post-fixed in 4% paraformaldehyde for 8 h. Next, they were cytoprotected by soaking in 10% and then 30% sucrose until sinking.25 Serial coronal sections were cut through the substantia nigra pars compacta (SNpc) at 20 μm by a freezing microtome. The sections were mounted on slides and stored at −20 °C until use.

Sections were rinsed several times in PBS. Tissue endogenous peroxidase was inactivated by incubating in 10% methanol and 3% hydrogen peroxide in PBS for 10 min. After three washes in PBS, the sections were pre-incubated in blocking buffer (PBS containing 10% goat serum) to reduce non-specific binding, and then were incubated overnight at 4 °C in a humidified chamber with rabbit TH primary antibody at dilution of 1 : 500. Sections were rinsed in PBS, then incubated with goat anti-rabbit secondary antibody for 30 min at 37 °C following the manufacturer’s instructions. The sections were stained with a dimethylaminobenzene (DAB) kit, dehydrated in graded alcohols, cleared with xylene and coverslipped. Control sections were treated with the same protocol but omitting the primary antibody. For morphological analysis, the images were recorded with an inverted microscope (OLYMPUS) connected to a camera. Cell counts were determined from 4 anatomically matched sections from each of the animals, and 3 animals were used for cell counts.

Assay of SOD Activity, GSH-PX Activity, MAO-B Activity and MDA Content  Four days after MPTP treatment, the brain was quickly removed, and the substantia nigra was isolated on an ice-cold glass plate. Samples were weighed accurately and prepared with 0.86% normal saline to give 10% tissue homogenate by super-audible cell disintegrator (Sonics), which was then centrifuged at 3000 rpm/min for 15 min at 4 °C. The supernatant was collected and kept at −80 °C.
until use. The protein concentration of the substantia nigra was determined by the method of Lowry et al. using bovine serine albumin as a standard.26)

With the respective detection kits (Nanjing Jiancheng Biotechnology, China), the activities of SOD, GSH-PX and MAO-B, and the content of MDA in the substantia nigra were determined following the kit specifications. Results are presented as units of activity per mg of protein (wet weight) or content.

**Statistical Analysis** Data are expressed as mean±standard deviation (S.D.). A one-way analysis of variance (ANOVA) followed by Dunnett’s post-hoc analysis was performed to determine whether individual doses were significantly different relative to controls. A p-value less than 0.05 was considered statistically significant.

**RESULTS**

**Motor Behavioral Test** In the rotarod test, the latent period that represented the time for mice to remain on the bar reduced significantly in the PD experimental model group, relative to the control group (p<0.01). The groups pre-treated with moderate and high doses of kaempferol and the positive control group (selegiline) rescued this reduction relative to the PD experimental model group (p<0.01, p<0.01, p<0.01, respectively, F5,30=11.170). Moreover, the effect of kaempferol in the spontaneous motor activity test was dose dependent. Results are shown in Fig. 2.

In the spontaneous motor activity test, the number of movements by the mice in 5 min reduced significantly in the PD experimental model group, relative to the control group (p<0.01). The groups pre-treated with moderate and high doses of kaempferol and the positive control group (selegiline) rescued this reduction relative to the PD experimental model group (p<0.05, p<0.01, p<0.01, respectively, F5,30=11.170). Moreover, the effect of kaempferol in the spontaneous motor activity test was dose dependent. Results are shown in Fig. 3.

**Striatal Dopamine and DOPAC Levels** The effects of kaempferol on the levels of dopamine and DOPAC in the striata of MPTP-induced PD model mice are shown in Table 1. The basal striatal levels of dopamine and DOPAC decreased in the MPTP experimental group relative to the control group (p<0.01 for both comparisons). Compared with the MPTP-treatment group, both the kaempferol pre-treatment groups and the selegiline pre-treatment group attenuated MPTP-induced dopamine depletion (p<0.01, p<0.05, p<0.01, p<0.01, respectively, F5,30=26.408). The groups pre-treated

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**Fig. 2.** The Time Period on the Rotarod

The duration of time on the rotating rod was significantly decreased by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment (15.2±5.7 s) when compared with the control group (102.0±16.7 s), but this difference was reversed by kaempferol pre-treatment (21.0±6.1 s, 50.8±10.2 s, 82.5±9.8 s, respectively) and selegiline (84.8±15.8 s). Values are presented as mean±S.D. (n=6). **p<0.01, compared with the MPTP group. ##p<0.01, compared with the control group.

**Fig. 3.** The Number of Total Movements in 5 min in the Spontaneous Locomotion Test

Locomotion counts were drastically reduced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment (145.3±31.0) when compared with the control group (275.3±41.4). However, this reduction in motor activity was prevented by kaempferol (182.7±39.5, 230.8±10.2 s, 242.0±35.8 s) and selegiline (256.0±34.0). Values are presented as means±S.D. (n=6). *p<0.05, **p<0.01, compared with the MPTP group. #p<0.01, compared with the control group.
with high doses of kaempferol showed reduced MPTP-induced DOPAC depletion \( (p<0.01, F_{5,30}^*=30.975) \). Moreover, in the kaempferol pre-treatment groups, the results were dose dependent.

The ratio DOPAC/dopamine, which is indicative of dopamine turnover in dopaminergic terminals, was increased in the experimental group \( (p<0.05) \), while it was altered by high doses of kaempferol and selegiline \( (p<0.05, p<0.01, \text{ respectively}, F_{5,30}^*=20.115) \) markedly. Results are shown in Fig. 4.

**Tyrosine Hydroxylase (TH) Immunohistochemistry**

Tyrosine hydroxylase staining was performed to evaluate the survival of dopaminergic neurons. Morphological observation was shown in Fig. 5A. In the control group, TH-positive cells were plentiful, cytoplasm and neurofibers of which were clear and intensively stained. Mice in the model group showed a pronounced reduction in the number of TH-positive cells with their neurofibers fractured and smaller cell size in comparison with the control group; no effect was seen in mice given kaempferol and selegiline, this administration resulted in an increase in TH-positive cells with similar cell morphology with the control group, thus suggesting kaempferol could protect dopaminergic neurons from MPTP neurotoxicity in mice.

In agreement with the above cellular morphological observation, in the control group, the average cell count was 86±10 per section. In MPTP group, the mean was 39±14 per section \( (p<0.01) \). In kaempferol (100 mg/kg) pre-treatment MPTP group, the mean was 76±10 per section \( (p<0.01) \). In the selegiline group pre-treatment MPTP group, the mean was 79±9 per section \( (p<0.01, F_{5,44}^*=46.666) \).

**Effects of Kaempferol on the Activity of SOD, GSH-PX and on the MDA Levels**

The effects of kaempferol on the activity of SOD, GSH-PX and the content of MDA in the substantia nigra of mice are shown in Table 2. MPTP administration in the PD model group mice resulted in a significant reduction in SOD and GSH-PX activity and increased level of MDA relative to the control group \( (p<0.01 \text{ for all comparisons}) \). However, this condition was partially rescued in the kaempferol-pre-treatment and selegiline groups. The activities of SOD in either kaempferol groups or the selegiline group were increased \( (p<0.01 \text{ for all comparisons}, F_{5,30}^*=52.192) \). The activities of GSH-PX were increased in the groups given moderate and high doses of kaempferol \( (p<0.01 \text{ for both comparisons}, F_{5,30}^*=33.06) \). The level of MDA was decreased in the moderate and high doses of kaempferol groups and selegiline group \( (p<0.01 \text{ for all comparisons}, F_{5,30}^*=65.256) \).

**Effects of Kaempferol on the Activity of MAO-B**

As shown in the Table 3, MPTP treatment induced a marked increase in MAO-B activity \( (p<0.01) \). Selegiline, the well known MAO-B inhibitor, exerted a strong effect on MAO-B inhibition \( (p<0.01) \). However, pre-treatment with kaempferol at either dose failed to significantly affect the activity of MAO-B \( (p>0.05, F_{5,30}^*=7.325) \), therefore suggesting that the neuroprotective effects of kaempferol was not due to the MAO-B inhibition.

### Table 1. Effects of Kaempferol on the Levels of Dopamine (DA) and 3,4-Dihydroxyphenylacetic Acid (DOPAC) in the Striata of Mouse 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) Model

<table>
<thead>
<tr>
<th>Group</th>
<th>DA (μg/g)</th>
<th>DOPAC (μg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.53±1.70</td>
<td>5.08±0.43</td>
</tr>
<tr>
<td>MPTP 30 mg/kg</td>
<td>4.48±0.86**</td>
<td>3.11±0.49**</td>
</tr>
<tr>
<td>Kaempferol (25 mg/kg)</td>
<td>6.81±0.75**</td>
<td>3.70±0.67</td>
</tr>
<tr>
<td>Kaempferol (50 mg/kg)</td>
<td>7.51±1.26*</td>
<td>3.91±0.15</td>
</tr>
<tr>
<td>Kaempferol (100 mg/kg)</td>
<td>9.25±1.36**</td>
<td>4.44±0.38***</td>
</tr>
<tr>
<td>Selegiline (15 mg/kg)</td>
<td>9.74±0.85**</td>
<td>2.18±0.40</td>
</tr>
</tbody>
</table>

Values are mean±S.D. \( (n=6) \), *\( p<0.05 \), **\( p<0.01 \), compared with the MPTP group. \( \star p<0.01 \), compared with the control group.

### Table 2. Effects of Kaempferol on the Activity of Superoxide Dismutase (SOD), Glutathione Peroxidase (GSH-PX) and the Content of Malondialdehyde (MDA)

<table>
<thead>
<tr>
<th>Group</th>
<th>SOD (U/mg prot)</th>
<th>GSH-PX (U/mg prot)</th>
<th>MDA (nmol/mg prot)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>160.33±15.31</td>
<td>24.17±3.31</td>
<td>6.12±0.29</td>
</tr>
<tr>
<td>MPTP 30 mg/kg</td>
<td>83.00±7.40**</td>
<td>9.00±1.78**</td>
<td>13.23±0.85**</td>
</tr>
<tr>
<td>Kaempferol (25 mg/kg)</td>
<td>106.33±8.58**</td>
<td>11.33±2.07</td>
<td>11.15±1.32</td>
</tr>
<tr>
<td>Kaempferol (50 mg/kg)</td>
<td>133.67±6.89**</td>
<td>15.50±1.52**</td>
<td>9.70±0.54**</td>
</tr>
<tr>
<td>Kaempferol (100 mg/kg)</td>
<td>152.83±9.64**</td>
<td>16.17±1.60**</td>
<td>8.75±0.36**</td>
</tr>
<tr>
<td>Selegiline (15 mg/kg)</td>
<td>146.17±10.83**</td>
<td>12.17±2.79</td>
<td>9.17±0.43**</td>
</tr>
</tbody>
</table>

Values are mean±S.D. \( (n=6) \), **\( p<0.01 \), compared with the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) group. \( \star p<0.01 \), compared with the control group.
DISCUSSION

Our studies showed that treatment with kaempferol improved motor abnormalities and increased striatal dopamine and DOPAC content in PD mice. Moreover, as a possible mechanism of its neuroprotection, kaempferol increased the activity of SOD and GSH-PX, reduced the content of MDA, which indicated its raised anti-oxidative capacity.

The neurotoxin MPTP is known to cause C57BL/6 mice to develop parkinsonism. The behavioral manifestation, neurochemical features and primary pathological condition induced by MPTP in mice are similar to that shown by PD patients. Therefore, MPTP-treated C57BL/6 mice make excellent conventional models and this regime is widely used for studies on PD.1,3,23) In the present study, MPTP-lesioned mice showed a behavioral deficit, but no mice were dead during the MPTP treatment. Kaempferol-pre-treated mice exhibited increased motor coordination and spontaneous locomotion compared with MPTP-treated mice alone.

Selegiline (L-deprenyl) is believed to render protection against MPTP neurotoxicity to a significant extent via a free radical scavenging mechanism and the ability to inhibit MAO-B in the brain. Its neuroprotective action was attributed to the inhibition of the metabolism of dopamine in the brain by dopamine reuptake inhibition, stimulation of antioxidant enzyme, such as SOD and catalase and increased turnover of dopamine, etc.27—29) Therefore, we use selegiline as a positive control to evaluate the neuroprotective effect of kaempferol.

Dopamine is the primary neurotransmitter involved in motor functions, its loss directly impacts physical movements and contributes to the clinical symptoms,2) depletion of which is also considered a cardinal feature in the cause of PD in humans or in animal models of the disease.30,31) MPTP causes a partial lesion of the substantia nigra and a significant reduction in striatal dopamine levels.23,32) Drugs that are able to ameliorate MPTP-induced neuronal damage are considered to be neuroprotective. The results of our present study show that the pre-intake of kaempferol markedly improved MPTP-induced dopamine and DOPAC depletion in the striatum, and reduced the DOPAC/dopamine ratio, which was significantly altered by MPTP. The metabolite/neurotransmitter ratio is an index of the rate of neurotransmitter metabolism. A decrease in the ratio indicates a decrease in neurotransmitter renovation rate.23,33) The enhancement of dopamine content by kaempferol might have restored the changes in locomotor activity. The neuroprotective effect of kaempferol may be due, in part, to a decrease in dopamine metabolism in the striata.

Dopamine is synthesized in two steps. Firstly, tyrosine is converted to L-dihydroxyphenylalanine (L-DOPA) by TH, and then L-DOPA is then converted to dopamine by L-DOPA decarboxylase. Tyrosine hydroxylase is, therefore, a key enzyme for dopamine biosynthesis and is used as a marker for dopaminergic neurons.34,35) Our results show that administration of kaempferol reduced the MPTP-induced loss of tyrosine hydroxylase-positive neurons in the mouse substantia nigra, which suggest that kaempferol may enhance the survival of dopamine neurons in MPTP-lesioned mice.

To further investigate the mechanism of action of kaempferol, we measured the anti-oxidative capacity and the activity of MAO-B. Oxidative stress refers to the cytologic consequences of a mismatch between the production of free...
radicals and the ability of the cell to defend against them. This imbalance results in apoptosis of neurons and auto-oxidation of dopamine. Our studies show a decrease in the activities of SOD and GSH-PX in the MPTP group. However, the decrease of antioxidant enzyme activities caused by MPTP was markedly restored by pre-treatment with kaempferol. Meanwhile, the level of MDA, which serves as an index for determining the extent of lipid peroxidation, was reduced by kaempferol which was altered by MPTP. Restoration of the activities of SOD and GSH-PX and reduction of the content of MDA due to pre-treatment with kaempferol demonstrate the protective role of kaempferol.

As previously reported, kaempferol behaves as a potent MAO inhibitor in vitro. It also has been shown that the MAO-B activity is elevated with age, which is directly related to PD, and MAO-B inhibitor has been used as a classic drug for clinical treatment. In the PD mouse model, MPTP was metabolized selectively by MAO-B to the active toxin MPP+. Sundstrom and Jonsson reported that MAO-B inhibitor could attenuate the MPTP-induced increase of dopamine turnover through inhibition of dopamine metabolism in the mouse. The activity of MAO-B, therefore, is of critical importance. We evaluated the activity of MAO-B in the mouse striata by exploring the action of kaempferol. Data obtained in the present experiments show that pre-treatment with kaempferol could not effectively inhibit MAO activity, but selegiline could, suggesting that kaempferol does not exert a MAO-B inhibiting effect in vivo. The two studies were different in methods and its sensitivity. Furthermore, kaempferol in the study of the reference cited produced more inhibition of MAO-A, not MAO-B. While in our study, we only detected the effect of kaempferol on MAO-B inhibition. Considering these differences, kaempferol didn’t have a MAO-B inhibition in our present study, unlike the results in reference. Or the number of samples was a little small. Thus, the excellent anti-oxidative capacity, but not MAO-B inhibition, contributed more to the neuroprotective mechanism of kaempferol.

In conclusion, our data provide evidence that kaempferol has neuroprotective effects in MPTP-induced PD mice, which may have contributed to its anti-oxidative capacity to scavenge free radicals and resulted in the survival of more dopamine neurons. We propose that kaempferol has shown anti-parkinsonian properties, pending future studies to elucidate further detailed mechanisms of action.

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REFERENCES