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Neuroprotective Effects of Longan (*Dimocarpus longan* Lour.) Flower Water Extract on MPP$^+$-Induced Neurotoxicity in Rat Brain

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ABSTRACT: In this study, the neuroprotective effect of *Dimocarpus longan* Lour. flower water extract (LFWE) was investigated. First, an in vitro study showed that LFWE concentration-dependently inhibited lipid peroxidation of brain homogenates incubated at 37 °C. The antioxidative activity of LFWE was more potent than that of glutathione or Trolox. Furthermore, an ex vivo study found that the basal lipid peroxidation (0 °C) and lipid peroxidation incubated at 37 °C were lower in the brain homogenates of LFWE-treated (500 mg/day) rats, indicating that the brain of LFWE-treated rats was more resistant to oxidative stress. Moreover, a Parkinsonian animal model was employed to demonstrate that oral administration of LFWE (125−500 mg/kg/day) dose-dependently attenuated 1-methyl-4-phenylpyridinium (MPP$^+$)-induced neurotoxicity in the nigrostriatal dopaminergic system of rat brain. In conclusion, this study suggests that LFWE is antioxidative, anti-inflammatory, and anti-apoptotic. Furthermore, oral administration of LFWE appears to be useful in preventing and/or treating central nervous system neurodegenerative diseases, including Parkinsonism.

KEYWORDS: *Dimocarpus longan Lour., MPP$^+$, neurotoxicity, oxidative stress, α-synuclein aggregation, anti-inflammation, anti-apoptosis*

INTRODUCTION

Nowadays, more and more studies have focused on neuroprotective strategies using dietary supplements for the prevention and/or treatment of neurodegenerative diseases in the central nervous system (CNS).[1−3] *Dimocarpus longan* Lour., a plant in the Sapindaceae family, is a very popular fruit in China and Southeast Asia. In Chinese traditional medicine, the flowers of longan have been used for the treatment of leucorrhea, kidney disorders, and lung disorders.[4] Recent studies have demonstrated that longan flowers may be beneficial against lipopolysaccharide-induced inflammation[5] as well as metabolic syndromes induced by fructose and a hypercaloric diet.[6,7] An abundance of active flavonoids, including proanthocyanidins, has been identified for the therapeutic effects of longan flowers via antioxidative mechanisms.[5,8,9]

Oxidative stress, neuroinflammation, and apoptosis are proposed as part of a vicious cycle in CNS neurodegeneration.[10−24] Clinical studies support this notion by demonstrating elevated lipid peroxidation and reduced antioxidative defense systems in the brains of patients with Parkinson's disease.[14,15] Many studies have employed transition metals and neurotoxins to confirm the pathological role of oxidative stress in Parkinsonism.[16−24] Several pathological features have been demonstrated, such as elevated lipid peroxidation and apoptotic neurons in Parkinsonian cell models.[16,17] Furthermore, behavioral abnormalities, reduced striatal dopamine content, and activated microglia in the affected nigrostriatal dopaminergic system were observed in Parkinsonian animals.[18−22] Our previous studies have shown that melatonin, carboxyfullerenes, and herbal drugs may be neuroprotective against the deteriorated nigrostriatal dopaminergic system in Parkinsonian animals.[18−20] In the present study, we evaluated the neuroprotective effects of *D. longan* Lour. flower water extract (LFWE), a dietary supplement containing natural antioxidants, such as (−)-epicatechin and proanthocyanidin A2,[5,7−9] in a Parkinsonian animal receiving intranigral infusion of 1-methyl-4-phenylpyridinium (MPP$^+$), a neurotoxin commonly used in Parkinsonian studies.[17,19,21−24] In the brain, MPP$^+$ is taken up by the dopamine-reuptake system and accumulates in the dopaminergic neurons.[21] The neurotoxic action of MPP$^+$ is reportedly mediated through oxidative mechanisms by inhibiting NADH−CoQ10 reductase (complex I) of the respiratory chain in mitochondria and then generating reactive oxygen species (ROS).[21,22] Our data showed that oral

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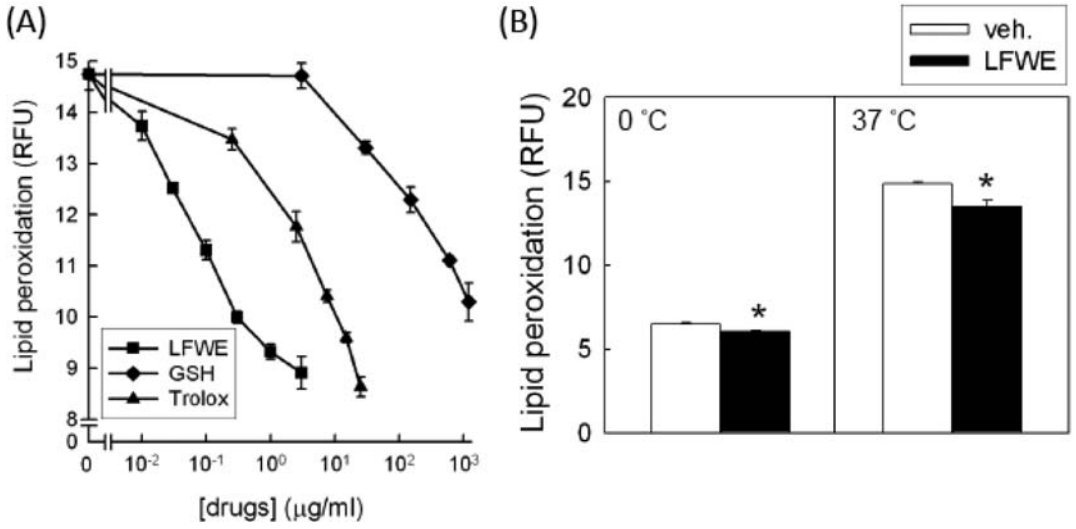

Figure 1. Antioxidative effects of LFWE on lipid peroxidation of brain homogenates in vitro and ex vivo. (A) Lipid peroxidation was performed in the brain homogenates incubated at 37 °C for 3 h with LFWE, glutathione (GSH), and Trolox. After incubation at 37 °C for 3 h, the lipid peroxidation was reported as relative fluorescence units (RFU). Values are the mean ± SEM (n = 3) from a representative experiment that was replicated with similar results. (B) Lipid peroxidation was performed in the brain homogenates from vehicle- or LFWE-treated rats. Values are the mean ± SEM (n = 6). After incubation at 0 °C (as basal levels) or 37 °C for 3 h, the brain lipid peroxidation was reported as RFU. ∗, $p < 0.05$ in the LFWE-treated rats compared with that in vehicle-treated rats by unpaired t test.

administration of LFWE appears to be neuroprotective via attenuating MPP^+-induced oxidative injury, α-synuclein aggregation, neuroinflammation, and apoptosis in the nigrostriatal dopaminergic system of rat brain.

MATERIALS AND METHODS

Adult, male Sprague−Dawley rats, weighing 250−350 g, were supplied by the National Laboratory Animal Breeding and Research Center, Taipei, Taiwan, ROC. All animals (3 rats/cage) were housed in an air-conditioned room (22 ± 2 °C) on a 12 h light/dark cycle (6:00 a.m.−6:00 p.m. light) and had free access to food and water. These animals were maintained according to the guidelines established in the *Guide for the Care and Use of Laboratory Animals* prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources Commission on Life Sciences, National Research Council, USA (1985). The use of animals has been approved by the Institutional Animal Care and Use Committee of Taipei Veterans General Hospital, Taipei, Taiwan, ROC.

In Vitro Studies Using Brain Homogenates. Brain samples were homogenized in chilled Ringer's solution (50 mg/mL) and incubated at 0 °C (as basal levels) or 37 °C for 3 h, and lipid peroxidation was measured using a spectrofluorometer (Hitachi F-4500, Hitachi High-Technologies Corp., Tokyo, Japan). To study the antioxidative activity of LFWE on lipid peroxidation, glutathione (Calbiochem, San Diego, CA, USA) and Trolox (Sigma, St. Louis, MO, USA) were used as positive controls. At the end of the experiment, a 400 μL sample was transferred to an eppendorf tube containing 300 μL of chloroform and 100 μL of methanol. The slurry was mixed and kept on ice for 15 min. After centrifugation (8000g for 5 min), an aliquot of chloroform extract was transferred to another tube containing 100 μL of methanol. Lipid peroxidation expressed as relative fluorescence units (RFU) was determined by measuring the levels of malondialdehyde and its dihydropyridine polymers, which emit fluorescence at 426 nm when activated by UV at 356 nm.[25]

Surgery and Intranigral Infusion of Drug. Rats were anesthetized with chloral hydrate (400 mg/kg, ip, Sigma, St. Louis, MO, USA) and placed in a stereotaxic instrument (David Kopf Instruments, Palo Alto, CA, USA). After skin incision and exposure of the parietal bone, holes above the cortical surface were drilled for intranigral infusion of drugs.[19,24] One microliter of MPP^+ (3 μg/μL, Sigma) was infused stereotaxically into the substantia nigra (SN) with coordinates of 3.2 mm anterior, 1.8 mm above the interaural zero, 2.1 mm lateral to the midline, and 3.5 mm below the incisor bar.[26] Drug solutions were infused at a rate of 0.2 μL/min through a 30 gauge stainless steel needle. The injection needle was held in place for an additional 3 min following drug infusion. After the surgery, rats recovered from anesthesia and were placed in home cages for the indicated times.

LFWE Preparation. Roasted male longan flowers were kindly provided by a certified longan farm (Tainan, Taiwan). Ground powder of longan flower was passed through a 40 mesh sieve and soaked with a 50 v/w (mL/g) ratio of distilled water at 100 °C for 5 min. To increase the extract efficiency, occasional shaking was performed. Afterward, the water extract was filtered through a no.1 filter paper and freeze-dried to obtain lyophilized powder; LFWE was then stored at −20 °C. The composition of LFWE has been identified previously.[8]

Oral Administration of LFWE. Rats were randomly divided in four groups. Three groups received LFWE at 125, 250, and 500 mg/kg/day, respectively, via an intragastric needle, and the fourth group received distilled H_2O as the control group 1 h prior to an intranigral infusion of MPP^+. Afterward, daily administration of LFWE continued as indicated for each experiment.

Fluorescence Assay of Lipid Peroxidation in SN. Dissected SN was homogenized in 400 μL of chilled chloroform and 200 μL of methanol. After centrifugation, an aliquot of the chloroform and methanol layer was scanned using a spectrofluorometer (Hitachi F-4500, Hitachi High-Technologies Corp.). Lipid peroxidation was determined by measuring the levels of malondialdehyde and its dihydropyridine polymers, which emit fluorescence at 426 nm when activated by UV at 356 nm.[25]

High-Pressure Liquid Chromatography Coupled with Electrochemical Detection (HPLC-ECD) Analysis of Striatal Dopamine Content. After decapitation, striata were dissected, immediately frozen in liquid nitrogen, and stored at −80 °C until analysis. An HPLC (CC5/PM80, Bioanalytical Systems Inc., West Lafayette, IN, USA) with electrochemical detection (LC-4C, Bioanalytical Systems Inc.) procedure was employed to measure striatal dopamine content. Applied potential was 0.75 V versus Ag/AgCl as reference. Mobile phase (1 L) contained 2.1 g of heptanesulfonic acid, 3.5 mL of triethylamine, 3 mL of phosphoric acid, 0.1 g of NaEDTA, and 170 mL of acetonitrile. The retention time for dopamine was about 7.5 min.[27]

Western Blot Analysis of Relevant Proteins. At the end of in vivo experiments, rats were sacrificed by decapitation. Dissected SN

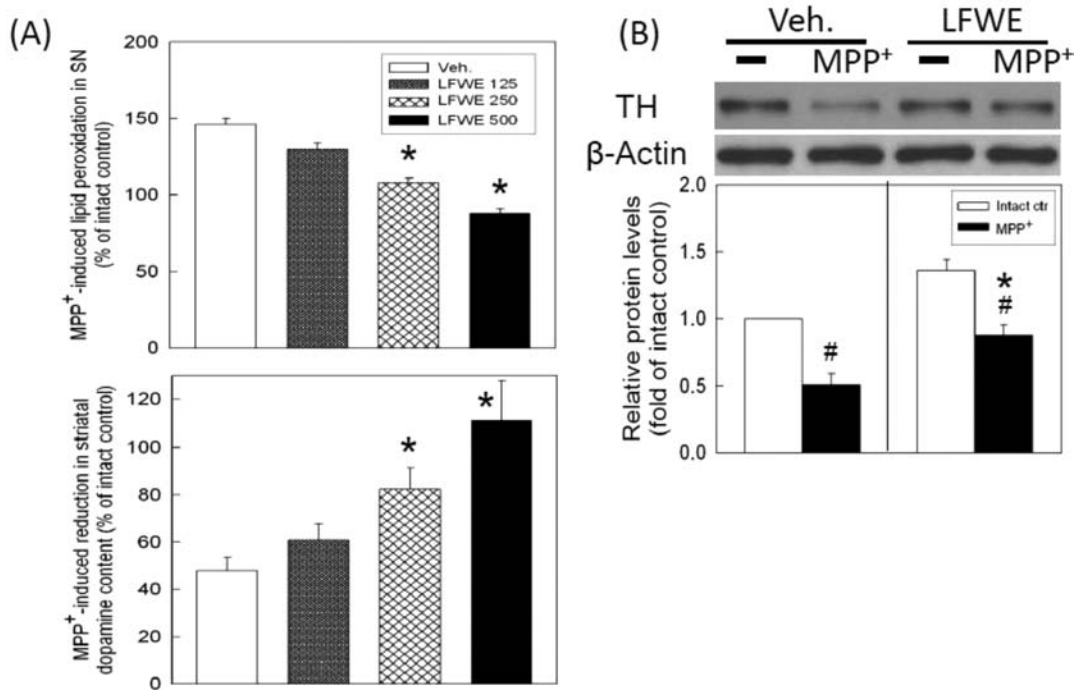

Figure 2. Effects of LFWE on MPP+-induced oxidative injury in the nigrostriatal dopaminergic system of rat brain. One hour prior to the intranigral infusion of MPP+ (3 μg/μL), LFWE (250 mg/kg) was administered orally. Afterward, LFWE was administered daily for 8 additional days. (A) Eight days after MPP+ infusion, oxidative injury was measured, including lipid peroxidation reported as relative fluorescence units (RFU) in the substantia nigra (SN) and striatal dopamine content detected by HPLC-ECD. Values are the mean ± SEM (n = 4−5). *, $p < 0.05$ in the MPP+-infused SN of LFWE-treated rats compared with that in vehicle-treated rats by unpaired t test. (B) Four days after MPP+ infusion, tyrosine hydroxylase (TH) immunoreactivity in the SN was evaluated using Western blot assay. Each lane contained 15 μg of protein for all experiments. Graphs show statistical results from relative optical density of bands on the blots estimated by Imagequant software. Values are the mean ± SEM (n = 3). #, $p < 0.05$ in the MPP+-infused SN compared with the intact SN; *, $p < 0.05$ in the MPP+-infused SN of LFWE-treated rats compared with that in vehicle-treated rats by unpaired t test.

was homogenized with a sonicator in 40 μL of ice-cold protease inhibitor cocktail (Calbiochem, San Diego, CA, USA). After homogenization, the lysates were centrifuged at 12000g at 4 °C for 30 min, and the supernatant was stored at −80 °C. Protein samples were run on 8−15% sodium dodecyl sulfate (SDS)−polyacrylamide gel electrophoresis and then transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) at 80 V for 120 min. Blots were probed with a monoclonal antibody against tyrosine hydroxylase (TH) (1:3000; Chemicon, Temecula, CA, USA), heme oxygenase-1 (HO-1) (1:3000; BD, Bedford, MA, USA), α-synuclein (1:3000; BD Transduction Laboratory, Lexington, KY, USA), ED-1 (1:1000; AbD Serotec, Raleigh, NC, USA), procaspase 12 (1:1000; Exalpha Biologicals, Shirley, MA, USA), and active caspase 3 (1:1000; Cell Signaling Technology, Beverly, MA, USA) at room temperature for 2 h. After primary antibody incubation, the membrane was washed and incubated with horseradish peroxidase-conjugated secondary IgG (1:3000; Chemicon) for 1 h at room temperature. The immunoreaction was visualized by Amersham enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA). After this detection, the bound primary and secondary antibodies were stripped by incubating the membrane in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS) at 50 °C for 45 min. The membrane was reprobed with a mouse β-actin antibody (1:5000; Millipore, Bedford, MA, USA). The densities of blots were analyzed using a scanning densitometer, which was operated by Scanner Control software (Molecular Dynamics, Sunnyvale, CA, USA). Results were obtained by calculating the density using Imagequant software (American Biosciences, Blauvelt, NY, USA) and reported as relative optical density of the specific proteins.

Statistics. Statistical comparisons were made as indicated using an unpaired Student's t test.

RESULTS

Antioxidative Effect of LFWE on Lipid Peroxidation: in Vitro and ex Vivo Studies. To study the antioxidative activity of LFWE, brain homogenates were used by incubating at 0 °C (as basal levels) or 37 °C for 3 h. Our in vitro data showed that the formation of peroxidized lipids increased from 6.5 ± 0.1 relative fluorescence units (RFU) at 0 °C to 14.9 ± 0.1 RFU at 37 °C (n = 3). Co-incubation with LFWE concentration-dependently attenuated lipid peroxidation (Figure 1A). The antioxidative activity of LFWE was compared with that of glutathione and Trolox, a water-soluble analogue of vitamin E. The antioxidative efficacy of LFWE was more potent than that of glutathione and Trolox in inhibiting lipid peroxidation (Figure 1A).

An ex vivo study was designed to further test the antioxidative activity of LFWE using brain homogenates prepared from rats treated with LFWE (500 mg/kg) or vehicle for 7days. After 3 h of incubation at 0 °C (as basal levels) or 37 °C, lipid peroxidations were lower in brain homogenates from LFWE-treated rats compared with those from the vehicle-treated rats (Figure 1B).

Neuroprotective Effect of LFWE: in Vivo Studies. To investigate the neuroprotective effect of LFWE, a Parkinsonian animal model using an intranigral infusion of MPP+ was established in the chloral hydrate-anesthetized rats. Eight days after intranigral infusion of MPP+, lipid peroxidation, a biomarker of free radicals to attack cells, was elevated in the infused SN (Figure 2A). Oral administration of LFWE (125−500 mg/kg/day) for 8 days inhibited MPP+-induced lipid

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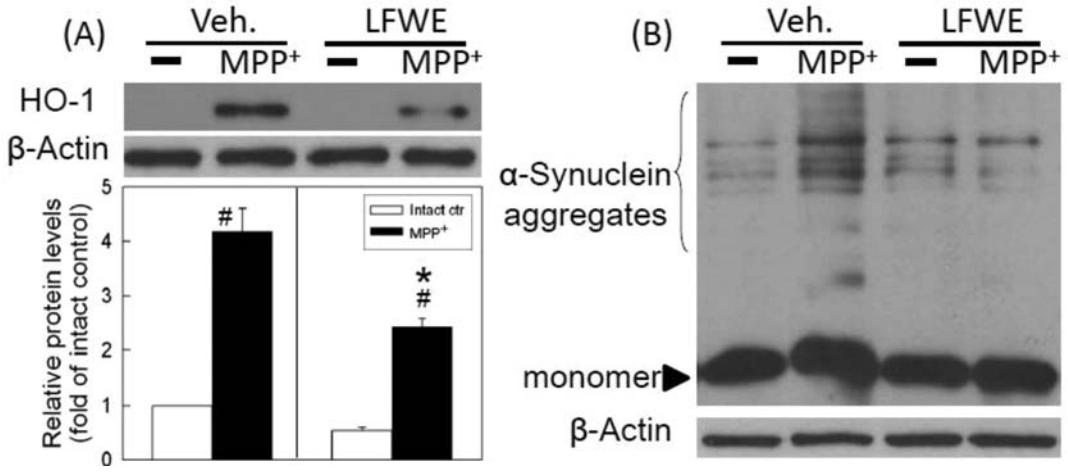

Figure 3. Effects of LFWE on MPP$^+$-induced elevations in HO-1 level and α-synuclein aggregation in the nigrostriatal dopaminergic system of rat brain. One hour prior to the intranigral infusion of MPP$^+$ (3 μg/μL), LFWE (250 mg/kg) was administered orally. Afterward, LFWE was administered daily. (A) Twenty-four hours after MPP$^+$ infusion, HO-1 levels in the substantia nigra (SN) were measured using Western blot assay. Each lane contained 30 μg of protein for all experiments. Graphs show statistical results from relative optical density of bands on the blots estimated by Imagequant software. Values are the mean ± SEM (n = 3). #, $p < 0.05$ in the MPP$^+$-infused SN compared with the intact SN; ∗, $p < 0.05$ in the MPP$^+$-infused SN of LFWE-treated rats compared with that in the vehicle-treated rats by unpaired t test. (B) Representative data show the effect of LFWE on α-synuclein aggregation using Western blot assay. Each lane contained 30 μg of protein for all experiments. Results were repeated in three independent experiments.

peroxidation in a dose-dependent manner. Medium and high doses of LFWE significantly attenuated MPP$^+$-induced lipid peroxidation in the infused SN. The integrity of nigrostriatal dopaminergic neurotransmission was studied by measuring dopamine content in the striatum and immunoreactivity of tyrosine hydroxylase (TH) in the SN. Using HPLC coupled with ECD, the dopamine content was reduced in the striatum ipsilateral to MPP$^+$-infused SN. Our data showed that LFWE dose-dependently attenuated MPP$^+$-induced reduction in striatal dopamine content (Figure 2A). Moreover, a Western blot assay demonstrated that LFWE (250 mg/kg/day) significantly prevented MPP$^+$-induced reduction in TH immunoreactivity (Figure 2B). These data indicate that LFWE can protect the nigrostriatal dopaminergic system from MPP$^+$-induced oxidative injury in the rat brain.

The cellular mechanisms underlying the LFWE-induced neuroprotection were investigated. Intranigral infusion of MPP$^+$ consistently elevated HO-1 levels, a sensor of oxidative stress, indicating that MPP$^+$ induced oxidative stress in the infused SN (Figure 3A). Compared with the vehicle-treated rats, the MPP$^+$-induced HO-1 elevation was significantly reduced in the LFWE-treated rats (Figure 3A). Furthermore, LFWE attenuated MPP$^+$-induced α-synuclein aggregation, a pathological feature of Parkinson's disease (Figure 3B), suggesting that LFWE is neuroprotective via reducing α-synuclein aggregation. The anti-inflammatory effect of LFWE was demonstrated by measuring ED-1, a biomarker for microglia activation. Our data showed that oral administration of LFWE significantly attenuated MPP$^+$-induced elevation in ED-1 level (Figure 4), indicating that LFWE prevented MPP$^+$-induced microglia activation in rat brain. In addition, our Western blot assay showed that LFWE profoundly inhibited MPP$^+$-induced reduction in procaspase 12 level (Figure 5A) and elevation in active caspase 3 level (Figure 5B), hallmark enzymes of apoptosis, suggesting an anti-apoptotic activity of LFWE in the rat brain.

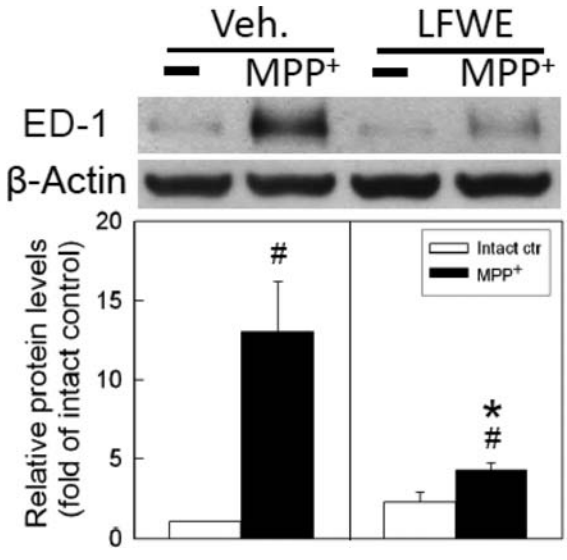

Figure 4. Effects of LFWE on MPP$^+$-induced microglia activation in the nigrostriatal dopaminergic system of rat brain. One hour prior to the intranigral infusion of MPP$^+$ (3 μg/μL), LFWE (250 mg/kg) was administered orally. Afterward, LFWE was administered daily. Four days after MPP$^+$ infusion, ED-1 levels in the substantia nigra (SN) were evaluated using Western blot assay. Each lane contained 30 μg of protein for all experiments. Graphs show statistical results from relative optical density of bands on the blots estimated by Imagequant software. Values are the mean ± SEM (n = 3). #, $p < 0.05$ in the MPP$^+$-infused SN compared with the intact SN; ∗∗, $p < 0.05$ in the MPP$^+$-infused SN of LFWE-treated rats compared with that in vehicle-treated rats by unpaired t test.

DISCUSSION

In the present study, LFWE was found to be neuroprotective against MPP$^+$-induced oxidative injury in the nigrostriatal dopaminergic system in several aspects. First, our in vitro and ex vivo data showed that LFWE is capable of inhibiting lipid peroxidation of brain homogenates, indicating that LFWE is

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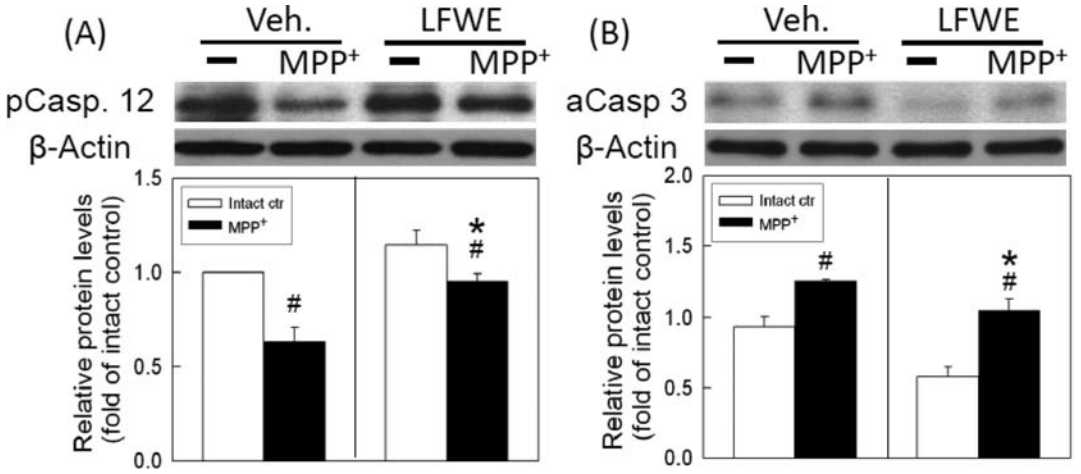

Figure 5. Effects of LFWE on MPP^+-induced reduction of procaspase 12 and elevation in active caspase 3 in the nigrostriatal dopaminergic system of rat brain. One hour prior to the intranigral infusion of MPP^+ (3 $\mu g/\mu L$), LFWE (250 mg/kg) was administered orally. Afterward, LFWE was administered daily. Four days after MPP^+ infusion, procaspase 12 (A) and active caspase 3 (B) protein levels in the substantia nigra (SN) were evaluated using Western blot assay. Each lane contained 30 μg of protein for procaspase 12 and 50 μg of protein for active caspase 3 experiments, respectively. Graphs show statistical results from relative optical density of bands on the blots estimated by Imagequant software. Values are the mean ± SEM ($n = 3$). #, $p < 0.05$ in the MPP^+-infused SN compared with the intact SN; *, $p < 0.05$ in the MPP^+-infused SN of LFWE-treated rats compared with that in the vehicle-treated rats by unpaired t test.

antioxidative. Furthermore, our in vivo study demonstrated that systemic administration of LFWE prevented MPP^+-induced oxidative injury by attenuating MPP^+-induced elevation in lipid peroxidation, HO-1 levels, and α-synuclein aggregation as well as MPP^+-induced depletion in striatal dopamine content. Moreover, LFWE was anti-inflammatory and anti-apoptotic against MPP^+-induced neurotoxicity, indicating that LFWE may be useful for preventing and/or treating CNS neurodegenerative diseases.

A significant number of studies have reported that plants with abundant polyphenols, such as green tea and longan flowers, are beneficial to human health.[1−3,5−9,20,28] Our previous studies support this notion: we found that green tea extract is neuroprotective by inhibiting iron-induced oxidative injury in rat brain.[28] Furthermore, LFWE, which contains significant amounts of antioxidative components, including total polyphenol (548.2 ± 12.7 mg/g), total flavonoids (139.3 ± 0.2 mg/g), and proanthocyanidins (112.5 ± 5.2 mg/g),[5] has been shown to exert its protective effect via antioxidative mechanisms.[5−8] To support this hypothesis, our in vitro data showed that LFWE reduced lipid peroxidation of brain homogenates. Furthermore, the antioxidative activity of LFWE was more potent than that of Trolox and glutathione. In addition, our ex vivo study showed that brain tissues from LFWE-treated rats were more resistant to oxidative stress. Due to the significant role of ROS in CNS neurodegenerative diseases,[10−16] our in vitro and ex vivo data strongly suggest that LFWE may be neuroprotective against the oxidative injury in CNS neurodegenerative diseases.

To evaluate the neuroprotective effect of LFWE, we chose an animal model of Parkinson's disease using intranigral infusion of MPP^+. Our study is the first to show that oral administration of LFWE is neuroprotective by inhibiting MPP^+-induced lipid peroxidation as well as MPP^+-induced reduction in striatal dopamine content and immunoreactivity of nigral TH, a rate-limiting enzyme of dopamine biosynthesis,[19,21] indicating that LFWE restored the MPP^+-deteriorated dopaminergic transmission in the nigrostriatal system of rat brain. In response to oxidative insults, HO-1, a chaperone protein, is reportedly elevated in cells to cope with unfavorable environments.[29,30] Our data showed that LFWE attenuated MPP^+-induced elevation in HO-1 levels, indicating that LFWE treatment reduced MPP^+-induced oxidative stress, and thus less HO-1 expression was observed. Microglia activation has been proposed as one of the sources of reactive oxygen/nitrogen species.[12,13] Furthermore, our previous study and others have reported that anti-inflammatory drugs including herbal drugs, which inhibit microglia activation, can be used as a potential therapy for CNS neurodegenerative diseases.[20,31−33] The present study showed that LFWE inhibited MPP^+-induced elevation in ED-1 levels, indicating that LFWE may exert its anti-inflammatory action by inhibiting MPP^+-induced microglia activation in the nigrostriatal dopaminergic system.

Clinically, elevated α-synuclein immunoreactivity was observed in Parkinsonian patients.[34,35] The proposed mechanism for elevation in α-synuclein aggregates is to induce endoplasmic reticulum (ER) stress and then activate unfolding protein response pathways (UPR),[36−38] including caspase 12 activation, an ER stress specific enzyme.[39] UPR activation reportedly reduces protein synthesis, facilitates protein refolding, and targets misfolded proteins for degradation.[36−38] However, abnormal ER stress may result in apoptotic cell death.[40,41] The present study showed that LFWE attenuated MPP^+-induced α-synuclein aggregates in the infused SN, indicating that LFWE may be neuroprotective via the inhibition of α-synuclein aggregates and then the reduction of ER stress. Indeed, LFWE restored procaspase 12 levels in the MPP^+-infused SN. Furthermore, LFWE prevented MPP^+-induced caspase 3 activation, indicating that LFWE is anti-apoptotic against MPP^+-induced neurotoxicity.

More and more studies have proposed antioxidative supplements as one potential neuroprotective strategy against oxidative injury in CNS neurodegenerative diseases. In the present study, both in vitro and ex vivo data suggest that LFWE is antioxidative. Furthermore, in vivo studies show that oral administration of LFWE is neuroprotective via antioxidative, anti-inflammatory, and anti-apoptotic mechanisms, suggesting

that LFWE may be used for preventing and/or treating CNS neurodegenerative diseases.

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Notes

The authors declare no competing financial interest.

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