Neuroprotective Effects of San-Jia-Fu-Mai Decoction: Studies on the in vitro and in vivo Models of Parkinson’s Disease

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Objective: The objective of this study was to investigate whether 70% aqueous ethanol extract of San-Jia-Fu-Mai decoction extract (SJFMDE) could protect against 1-methyl-4-phenylpyridinium (MPP+)-induced oxidative stress in PC12 cells and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced motor function deficits in mice. Materials and Methods: The cell viability, the levels of intracellular reactive oxygen species (ROS), malondialdehyde (MDA), and glutathione (GSH) in the MPP+-treated PC12 cells were measured. Motor function deficits and dopamine (DA) level in the brain striatum and tyrosine hydroxylase (TH)-positive cells in substantia nigra pars compacta (SNc) of the MPTP-treated mice were determined. Results: The results showed that SJFMDE could reduce cell death and the levels of ROS and MDA while increase the level of GSH in the MPP+-treated PC12 cells. In addition, in vivo studies showed that oral administration of SJFMDE (3, 6, and 12 g/kg) significantly improved the motor function deficits induced by MPTP and enhanced the DA level in the striatum and TH-positive neuronal cells in SNc of the MPTP-treated mice. Conclusions: Our results revealed that SJFMDE possessed neuroprotective effects against neurotoxicity induced by MPP+ and motor function deficits induced by MPTP via suppressing oxidative stress and increasing the levels of DA and TH, indicating that SJFMDE might be a promising Chinese medicine formula for the treatment of Parkinson’s disease.

Keywords: 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine, 1-methyl-4-phenylpyridinium, motor function deficits, oxidative stress, Parkinson’s disease, San-Jia-Fu-Mai decoction

INTRODUCTION

Parkinson’s disease (PD), one of the common neurodegenerative disorders, is characterized by motor symptoms of rigidity, tremors, bradykinesia, and postural instability. As one of the fastest growing neurological disorders in the world, it is estimated that the number of individuals affected by PD will double to approximately 13 million in 2040. To date, levodopa (L-DOPA) remains the gold standard for the treatment of PD. Personalized medicine for PD treatment has been an evolving research area in recent years, focusing particularly on the role of neurotransmitter systems in the motor and the nonmotor symptoms of PD. It has come to a revolutionary change of understanding that not only the dopaminergic pathway plays an important role, but the noradrenergic, serotonergic, and cholinergic pathways are also actively involved in the pathogenesis of PD. Four subtypes of PD, namely the dopaminergic subtype, the noradrenergic subtype, the serotonergic subtype, and the cholinergic subtype, each with different manifestations of nonmotor symptoms, require different individualized treatment strategies.

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At present, the pathogenesis of PD was not fully understood, and no animal model can imitate all pathological characteristics of PD patients. Oxidative stress plays a crucial role in the development of PD. Behavioral alterations such as tremors, bradykinesia, rigidity, and postural instability are the major clinical symptoms of PD. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a lipophilic compound, can pass through the blood–brain barrier and be metabolized by monoamine oxidase B into 1-methyl-4-phenylpyridinium (MPP+) to generate free radicals to induce PD-like symptoms in animals. Mice receiving acute or subacute injection of MPTP could replicate almost all of the pathological hallmarks of PD patients. Over the past decades, the MPTP-induced mouse PD model has been widely used in the studies for the development of preventive and therapeutic agents for AD.

San-Jia-Fu-Mai decoction (SJFMD), a Chinese medicine formula first recorded in a famous Chinese medicine book named “The Itemized Differentiation of Warm Diseases” (Wen-Bing-Tiao-Bian in Chinese) written by the venerated physician Wu Jutong of the Qing dynasty, is composed of nine herbs, i.e., Testudinis Carapax et Plastrum (Guiban), Trionycis Carapax (Biejia), Rehmanniae Radix (Dihuang), Paoniae Radix Alba (Baishao), Glycyrrhizae Radix et Rhizoma (Zhigancao), Ophiopogonis Radix (Maidong), Ostreeae Concha (Muli), Cannabis Fructus (Maziren), and Asini Cori Colla (Ejiao) at a ratio of 10:8:6:6:6:6:6:6:6:5. It has the therapeutic functions of nourishing Yin, clearing heat, calming wind, and nourishing the liver and kidney. In Chinese medicine practice, SJFMD is frequently prescribed to treat Parkinson’s disease (PD) and attention-deficit hyperactivity disorder. Recent studies have revealed that SJFMD has good anti-PD effects in animal models of PD via inhibiting the protein expression of alpha-synuclein in substantia nigra and striatum and ameliorating the damage of this brain region of the rotenone-treated rats. In a clinical trial comparing the yield of which was 25.62%. The SJFMD extract (SJFMDE) was stored at −20°C for further study.

Preparation of San-Jia-Fu-Mai decoction extract

The individual component herbal materials of SJFMD were purchased from Zhixin Herbal Pharmaceutical Company Ltd., a Guangzhou-based GMP-accredited Chinese herbal supplier, and authenticated by Ms. Yu-Ying Zong, who is a seasoned pharmacognost at the School of Chinese Medicine, The Chinese University of Hong Kong, Hong Kong SAR, China. The voucher specimens were conserved at the School of Chinese Medicine, The Chinese University of Hong Kong, with reference no. SJFM181101-181109. The mixture of SJFMD was extracted under reflux using 70% aqueous ethanol for 1 h. The extract was filtered, and the residue was further extracted twice as before. All three filtrates were combined, concentrated in a rotary evaporator under negative pressure, and finally dried in a lyophilizer to get a freeze-dried extract, the yield of which was 25.62%. The SJFMD extract (SJFMDE) was dissolved in dimethyl sulfoxide (DMSO) and placed in an ultrasonic bath until the solute was completely dissolved in the medium, followed by serial dilution to different concentrations with DMEM. To determine the toxicity of MPP+ or SJFMD, PC12 cells were treated with different concentrations of MPP+ (100, 200, 400, 600, and 800 μM) or SJFMD (0, 31.25, 62.5, 125, 250, 500, 1000, and 2000 μg/mL) for 24 h. To determine the neuroprotective effect of SJFMD, PC12 cells were pretreated with SJFMD (250, 500, and 1000 μg/mL) for 2 h, and then, MPP+ (final concentration of 800 μM) was added and incubated for an additional 22 h. The nontreated control was incubated with DMEM in the same way without MPP+ or SJFMD. The final concentration of DMSO in all culture wells was no more than 0.01%, and no cytotoxicity by DMSO was observed (data not shown).
Cell viability assay

Cell viability in the presence of MPP+ and SJFMDE was determined by MTT assay as described in our previous study. Briefly, after drug treatment, MTT assay was performed to determine the cell viability. Briefly, 20 μL of MTT solution (6 mg/mL) in phosphate-buffered saline (PBS) was added into each well and incubated at 37°C for 4 h. Following the aspiration of supernatants, 100 μL of DMSO was added to dissolve the insoluble purple formazan crystals into a colored solution, and its absorbance at 570 nm was then determined using a BioTek microplate reader (Winooski, VT, USA). The cell viability was shown as the percentage of the control group.

Determination oxidative stress in 1-methyl-4-phenylpyridinium-treated PC12 cells

Determination of intracellular reactive oxygen species production

The level of intracellular ROS was determined using the DCFH-DA method as described in our previous study. Briefly, DCFH-DA, a nonfluorescent compound, can be enzymatically converted to DCF, a highly fluorescent compound, in the presence of ROS. Briefly, after drug treatment, the cells were washed with PBS solution and incubated with DCFH-DA (10 μM) at 37°C for 30 min in dark. In order to remove the extracellular DCFH DA, the cells were washed twice with PBS solution. Afterward, the fluorescence intensity of DCF was determined using a FLUOstar OPTIMA microplate reader (BMG Labtech, Offenburg, Germany) with an excitation wavelength at 485 nm and an emission wavelength at 538 nm. The production of intracellular ROS was shown as the percentage of the control group.

Measurement of glutathione and malondialdehyde levels

The PC12 cells were plated onto a 100-mm² dish at 5 × 10⁶ cells/dish. The cells were washed with D-Hanks solution, then scraped from the dish into 1 mL cold PBS (0.1 M, containing 0.05 mM EDTA), and homogenized on ice after drug treatment for 24 h. The homogenate was centrifuged at 4000 g for 30 min at 4°C. The supernatants were harvested and stored at −80°C for further analyses. Protein concentration was determined by Bradford method using bovine serum albumin as a standard. MDA level was determined as per our previous methods. Briefly, an aliquot (100 μL) of supernatant was combined with 1.5 mL thiobarbituric acid (0.8%, w/v), 1.5 mL acetic acid (20% v/v, pH 3.5) and 0.2 mL sodium dodecyl sulfate (8%, w/v). All reaction mixture was incubated at 95°C for 60 min and then cooled to room temperature. Subsequently, each reaction solution was added with 5.0 mL of n-butanol and fully mixed. The mixture was then centrifuged at 3000 g at 4°C for 10 min, and the organic layer was harvested, and the absorbance was determined at 532 nm. The level of intracellular ROS was determined using the DCFH-DA method as described in our previous study.

Intraperitoneal injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in mice and drug treatment

To study the motor performance deficit ameliorating effects of SJFMDE in vivo, we established a MPTP-treated mouse model of PD as previously described. Briefly, C57BL/6 mice were randomly divided into six groups (n = 10): (a) control group; (b) MPTP group; (c-e) MPTP + SJFMDE (3, 6, and 12 g/kg) group, and (f) MPTP + rasagiline (10 mg/kg) group. The dose of rasagiline was selected from previous study. MPTP and rasagiline were dissolved in physiological saline. Mice were administered with MPTP (30 mg/kg) through intraperitoneal injection every morning for 7 consecutive days. SJFMDE was suspended in 0.5% sodium carboxymethyl cellulose and given intragastrically 2 h before MPTP injection for 7 days. Rasagiline was given to mice through subcutaneous injection 2 h before MPTP injection for 7 days, while the mice in the control group received the same volume of saline. One hour after the last dosing of MPTP, behavior tests on the mice were conducted. The experimental design and schedule are shown in Figure 1.

Behavior tests

Pole test

One hour after the last dosing of MPTP, the pole test was used to assess movement disorders of the PD mice. The animals were placed head-up on top of a vertical wooden pole (8 mm wide and 55 cm high) with a rough surface. The base of the pole was placed in the home cage. The animals placed on top of the pole turned downward and descended the pole back into their home cage. Each mouse was trained and accustomed to the test 1 day before the test. The total time for descending the pole (T-total) and the time for turning downward (T-turn) after each animal reached the cage floor with four paws were measured in a total of three attempts. A default value of 60 s was used for the mice unable to complete the test successfully.
Catalepsy test
Catalepsy is the inability of an animal to correct an externally imposed posture. Twenty-four hour after pole test, the catalepsy test was conducted as described in a previous study. Catalepsy test was measured after both forepaws of the mouse placing on a horizontal wooden bar (65 cm in length and 0.2 cm in diameter) and placing 15 cm above the floor. The time during the mice kept this position until raising their hindpaws onto the bar was recorded. This experiment was repeated six times for each mouse and the mean value calculated.

Tissue preparation
After behavioral tests, the mice were sacrificed by terminal anesthesia and perfused via intracardial infusion with saline, followed by 4% paraformaldehyde (pH 7.4). Brains were collected, then fixed in 4% paraformaldehyde for 24 h at 4°C, then dehydrated with graded ethanol, passed through chloroform, and embedded in paraffin for further immunohistochemical studies. The other part of tissues was immediately dissected out on ice and put into liquid nitrogen and then stored at −80°C for further immunoblotting analysis.

Immunohistochemistry assay
The brains of the mice were removed and fixed by 4% paraformaldehyde. Using a frozen microtome, the brains were cut into 8 μm sections. The sections were blocked in 5% BSA at room temperature for 20 min. Sections were incubated with anti-TH polyclonal antibody (Cat. No: ab137869, Abcam) at 4°C overnight. Then, the sections were incubated with fluoresence-conjugated secondary antibody at room temperature for 2 h. Immunofluorescent images were captured using a Zeiss fluorescent inverted microscope (Zeiss, Gottingen, Germany). The quantification of the TH-positive cells in SNC of mice was analyzed using Image J software (NIH, Bethesda, MD, USA).

Measurement of the dopamine level in the striatum of the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice
The striatum of mice was homogenized in 0.1 mL PBS. After incubating for 15 min on ice, the homogenates were centrifuged at 5000 rpm for 15 min at 4°C. The level of DA (Cat.: MBS732020) in the supernatants was determined using commercially available sandwich ELISA kits (MyBioSource, CA, USA) according to the manufacturer’s protocols. Briefly, 100 μL of standards or samples was added to the appropriate well. Then, 50 μL of conjugate solution was added into each well after mixing well and incubated for 1 h at 37°C. After washing the plate with 1 × wash solution five times, 50 μL Substrate A and 50 μL Substrate B were added into each well and reacted at 37°C for 20 min in dark. After adding stop solution (50 μL) to each well, the absorbance was measured at 450 nm using a FLUOstar OPTIMA microplate reader. The level of DA was normalized to the protein concentration of each sample and shown as percentage of the control group.

Statistical analysis
Data were shown as means ± standard error mean. The comparisons of multiple group were analyzed with one-way analysis of variance followed by Dunnett’s test. The statistical analysis was conducted using GraphPad Prism software (version 5, GraphPad Software, Inc., CA, USA). Statistically significant was considered when P < 0.05.

RESULTS

Effects of 1-methyl-4-phenylpyridinium and San-Jia-Fu-Mai decoction extract on the cell viability of PC12 cells
In order to explore the cytotoxicity of MPP+ and SJFMDE on PC12 cells, the cells were incubated with various concentrations of MPP+ or SJFMDE for 24 h. The results indicated that MPP+ reduced the cell viability in a dose-dependent manner. As shown in Figure 2a, treatment with MPP+ (400, 600, and 800 μM) for 24 h significantly decreased the cell viability in PC12 cells to 73.1%, 63.6%, and 48.7% (P < 0.001 for three doses), respectively, of the nontreated control (100%). On the other hand, SJFMDE at the concentrations up to 1000 μg/mL did not exhibit significant cytotoxicity to PC12 cells after incubation for 24 h [Figure 2b].

Effects of San-Jia-Fu-Mai decoction extract against the neurotoxicity induced by 1-methyl-4-phenylpyridinium in PC12 cells
PC12 cells were incubated with SJFMDE (250, 500, and 1000 μg/mL) for 2 h, followed by exposure to 800 μM of MPP+ for another 22 h. As shown in Figure 3, after pretreatment with SJFMDE (500 and 1000 μg/mL), the cell viability was significantly increased to 70.0% (P < 0.01) and 84.0% (P < 0.001), respectively, as compared with the vehicle-treated control group, revealing that SJFMDE exerted protection against MPP+-induced neurotoxicity in PC12 cells.

Effects of San-Jia-Fu-Mai decoction extract against oxidative stress induced by 1-methyl-4-phenylpyridinium in PC12 cells
As shown in Figure 4, oxidative stress induced by MPP+ in PC12 cells was measured by determining the levels of intracellular ROS [Figure 4a], MDA [Figure 4c], and GSH [Figure 4b]. After exposure of PC12 cells to 800 μM MPP+ for 24 h, intracellular ROS and MDA levels were markedly enhanced to 217% (P < 0.001) and 204% (P < 0.001), respectively, of the control value (100%), while GSH level was significantly
reduced to 58% \((P < 0.001)\) of the control value (100%), indicating that MPP+ could induce remarkable oxidative stress. When PC12 cells were pretreated with SJFMDE (500 and 1000 μg/mL) for 2 h, followed by exposure to 800 μM of MPP+ for another 22 h, intracellular ROS production was substantially reduced 154% and 126% of the control value, \(P < 0.01\) and \(P < 0.001\), respectively, as compared to the MPP+ group. Pretreatment with SJFMDE (500 and 1000 μg/mL) also significantly reduced MDA level (161% and 135% of the control value, \(P < 0.001\) and \(P < 0.001\), respectively) as compared to the MPP+ group. Pretreatment with SJFMDE (1000 μg/mL) significantly increased the GSH level (84% of the control value, \(P < 0.01\)) as compared to the MPP+ group. These findings demonstrated that SJFMDE could effectively ameliorate the MPP+-induced oxidative stress in PC12 cells.

**Effects of San-Jia-Fu-Mai decoction extract on motor function deficits induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in mice**

Motor deficits including bradykinesia, postural instability, and rigidity are the main clinical symptoms of PD patients. Two behavioral tests, including pole test and catalepsy test, were used to assess the improving motor function impairment effects of SJFMDE. As shown in Figure 5, the time that the mice took to climb from the top of the pole to the ground in the MPTP group was significantly longer than that in the control group. SJFMDE (6 and 12 g/kg) significantly shortened the descending time \((P < 0.05\) and \(P < 0.01\), respectively) in the MPTP-treated mice as compared to the MPTP-treated control group [Figure 3]. In the catalepsy test, the duration of immobility onto the bar in the catalepsy test in the MPTP group was longer than that in the control group \((P < 0.01\) ). Treatment with SJFMDE (3, 6, and 12 g/kg) reduced the duration of immobility onto the bar \((P < 0.05, P < 0.05,\) and \(P < 0.01,\) respectively) in the MPTP-treated mice as compared to the MPTP-treated control group [Figure 5b]. Moreover, rasagiline (10 mg/kg) also effectively reduced the descending time in the pole test \((P < 0.01)\) and the duration of immobility onto the bar in the catalepsy test \((P < 0.01)\) in the MPTP-treated mice.

**Effects of San-Jia-Fu-Mai decoction extract on the level of dopamine in the striatum of the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice**

The content of DA in the striatum of the MPTP-treated mice was measured by ELISA kit. As shown in Figure 6, MPTP treatment could significantly reduce the DA level \((P < 0.001)\) in the striatum of mice as compared to the control group. However, treatment with SJFMDE (6 and 12 g/kg) significantly elevated the DA level \((P < 0.05\) and \(P < 0.001,\) respectively) in the striatum of the MPTP-treated mice as compared to the MPTP-treated group. Similarly, rasagiline (10 mg/kg) also
effectively increased the DA level ($P < 0.05$) in the striatum of the MPTP-treated mice.

**Effects of San-Jia-Fu-Mai decoction extract on the tyrosine hydroxylase-positive neuronal cells in the substantia nigra pars compacta of the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice**

As shown in Figure 7, MPTP treatment significantly attenuated the number of TH-positive neuronal cells in the SNc of mice as compared to the control group ($P < 0.001$). Treatment with SJFMEDE (3, 6, and 12 g/kg) markedly enhanced the number of TH-positive neuron cells in the SNc of mice ($P < 0.001$, $P < 0.05$, and $P < 0.01$, respectively) as compared to the MPTP-treated control group. Likewise, rasagiline (10 mg/kg) also markedly increased the number of TH-positive neuron cells ($P < 0.05$) in the SNc of the MPTP-treated mice.

**DISCUSSION**

SJFMD is one of the most famous TCM formulae commonly prescribed by TCM practitioners to treat brain-related diseases such as stroke, Alzheimer’s disease, and PD. In this study, we for the first time investigated the protective effects of SJFMDE on the *in vitro* and *in vivo* models of PD. The results demonstrated that SJFMDE alleviated the MPP$^+$-induced cytotoxicity in PC cells via inhibition of the oxidative stress. In addition, SJFMDE effectively improved the motor functions and prevented the decrease of the TH protein level in SNc and DA level in the striatum of the MPTP-treated mice.

MPTP is converted to MPP$^+$ in the glia via monoamine oxidase B. MPP$^+$ can cross the blood–brain barrier and is selectively transported into dopaminergic neurons in the SNc, thereby damaging the dopaminergic neurons in the SNc.$^{18,19}$ Hence, the MPTP administration caused a reduction in the density of the TH-positive neurons and the level of DA and its metabolites in mice.$^{7,8}$ Our results revealed that SJFMDE effectively ameliorated the motor function deficits and enhanced the TH-positive cells in the SNc and the DA level in the striatum of the MPTP-treated mice. Lower DA may turnover resulted in lowering the level of ROS, devoting to the neuroprotective effect of SJFMDE.
Increasing evidence revealed that oxidative stress is a major biochemical pathogenic factor of PD, which is a result of a metabolic imbalance between the production and neutralization of ROS.\cite{20} It is also the imbalance between the intracellular free radicals levels and the intracellular antioxidant defense system activities.\cite{8,21} The brain consumes more oxygen per volume of tissue compared to the other organs of the body.\cite{22} Overproduction of ROS in neuronal cells would increase the oxygen free radicals in dopaminergic cells. The excessive oxygen free radicals can damage lipid membranes, interfere with cellular respiration, and interrupt DNA integrity by altering mitochondrial complex I.\cite{23} Excessive intracellular free radicals also assault the adjacent biomolecules, such as nucleic acids, polyunsaturated fatty acids, and proteins, and undermine the activities of the intracellular antioxidant defense systems such as catalase (CAT), superoxide dismutase (SOD), and the level of GSH. Once MPP⁺ is released into PC12 cells, MPP⁺ can enter into the mitochondria and then lead to the production of oxygen free radicals to form hydrogen peroxide and hydroxyl radicals, thereby causing oxidative damage in PC12 cells.\cite{24,25} Our results were consistent with the previous studies that MPP⁺ increased the levels of ROS and MDA but decreased in the level of GSH in PC12 cells, while SJFMDE treatment caused increases in the expression level of GSH content, accompanied by significant decreases in the levels of MDA and ROS. Therefore, SJFMDE prevents the neurotoxicity induced by MPTP in dopaminergic neurons via inhibiting oxidative stress. Total glucosides of *Paeoniae Radix Alba* has been reported to ameliorate the oxidative stress caused by *Strychnos* alkaloids in mice and in the corticosterone-treated PC12 cells via increasing the level of GSH and the activities of SOD and CAT.\cite{27,28} Extract of *Cannabis Fructus* could improve the cognitive deficits induced by D-galactose in rats by enhancing antioxidant capacity.\cite{29} These components in SJFMDE may act synergistically to provide anti-oxidative effect.

**Conclusions**

SJFMDE exerted significant neuroprotective effects against the MPP⁺-induced neurotoxicity via inhibition of oxidative stress, and markedly ameliorated motor function deficits induced by MPTP via elevating the number of TH positive cells in the SNC and the level of DA in the striatum of the MPTP-treated mice. Our experimental findings amply indicated that SJFMD...
is a herbal formula with potent neuroprotective property. The results from this project also helped put the application of this common herbal formula in TCM clinical practice for the treatment of PD on a scientific footing.

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Conflicts of interest
There are no conflicts of interest.

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Neuroprotective effects of San-Jia-Fu-Mai decoction in Parkinson’s disease


