

# Effects of Brain Factor-7<sup>®</sup> against motor deficit and oxidative stress in a mouse model of MPTP-induced Parkinson's disease

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**Abstract.** Oxidative stress is strongly implicated in the pathogenesis of Parkinson's disease (PD) through degeneration of dopaminergic neurons. The present study was designed to investigate the underlying mechanisms and therapeutic potential of Brain Factor-7<sup>®</sup> (BF-7<sup>®</sup>), a natural compound in silkworm, in a mouse model of PD induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP (20 mg/kg) was intraperitoneally injected into mice to cause symptoms of PD. Mice were orally administered BF-7<sup>®</sup> (a mixture of silk peptides) before and after MPTP treatment. Rotarod performance test was used to assess motor performance. Fluoro-Jade B staining for neurons

undergoing degeneration and immunohistochemistry of tyrosine hydroxylase for dopaminergic neurons, 4-hydroxy-2-nonenal (4HNE) for lipid peroxidation, 8-hydroxy-2'-deoxyguanosine (8OHdG) for DNA damage and superoxide dismutase (SOD) 1 and SOD2 for antioxidative enzymes in the pars compacta of the substantia nigra were performed. Results showed that BF-7<sup>®</sup> treatment significantly improved MPTP-induced motor deficit and protected MPTP-induced dopaminergic neurodegeneration. Furthermore, BF-7<sup>®</sup> treatment significantly ameliorated MPTP-induced oxidative stress. Increased 4HNE and 8OHdG immunoreactivities induced by MPTP were significantly reduced by BF-7<sup>®</sup>, whereas SOD1 and SOD2 immunoreactivities decreased by MPTP were significantly enhanced by BF-7<sup>®</sup>. In conclusion, BF-7<sup>®</sup> exerted protective and/or therapeutic effects in a mouse model of PD by decreasing effects of oxidative stress on dopaminergic neurons in the substantia nigra pars compacta.

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**Abbreviations:** 4HNE, 4-hydroxy-2-nonenal; 8OHdG, 8-hydroxy-deoxyguanosine; BF-7<sup>®</sup>, Brain Factor-7<sup>®</sup>; F-J B, Fluoro-Jade B; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease; ROD, relative optical density; ROS, reactive oxygen species; SOD, superoxide dismutase; SNpc, pars compacta of substantia nigra; TH, tyrosine hydroxylase

**Key words:** 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, dopamine, silk peptides, substantia nigra, striatum, tyrosine hydroxylase

## Introduction

Parkinson's disease (PD) is a prevalent neurodegenerative disorder with defective motor function, which is characterized by a progressive loss of dopaminergic neurons located in the pars compacta of the substantia nigra (SNpc) with subsequent exhaustion of dopamine in the striatum (1). PD has become increasingly common with advance in age. It affects 2-3% of the population aged more than 65 years (2). The main symptoms of PD are disabilities of the motor system, including akinesia, resting tremor, bradykinesia and rigidity (3,4).

Oxidative stress is the result of many metabolic processes essential to the body. However, it can exert toxic and deleterious roles in the body (5,6). During the pathogenesis of PD, oxidative stress is caused by dramatical increase in the concentration of reactive oxygen species (ROS) in cells, attacking macromolecules such as DNA, proteins and lipids, triggering a redox state-sensitive pro-death signaling pathway and ultimately leading to death of dopaminergic neurons in the SNpc (7). A

postmortem study on brains of Parkinson's disease patients has shown that dopaminergic neurons in the SNpc are degenerated and protein oxidation is elevated in the caudate nucleus (8). In addition, an *in vivo* study on patients with PD showed a significant increase in SOD activity in peripheral blood parameters in comparison with controls, suggesting that an important role of oxidative stress in PD evolution and progress (9).

Studies have demonstrated that various kinds of natural resources with significant antioxidant activities are effective against PD (10-12). BF-7<sup>®</sup> was developed and provided by Famenity Co., Ltd. (Uiwang, Gyeonggi, Republic of Korea). It is a mixture of silk peptides (~85% are peptides and alanine and tryptophan are included as major components) obtained from hydrolyzed fibroin of the cocoon shell of silkworm (*Bombyx mori*). Some studies using BF-7<sup>®</sup> have demonstrated that amyloid  $\beta$ -induced apoptosis in the SKN-SH cells (human neuroblastoma cell line) is inhibited and cognitive function in healthy subjects is increased (13,14). In addition, BF-7<sup>®</sup> plays positive roles in learning and memory deficits. It can attenuate ischemic brain damage in experimental ischemic stroke (15).

To the best of the authors' knowledge, neuroprotective effects of cocoon hydrate such as BF-7<sup>®</sup> against PD has been poorly investigated. Thus, the aim of the present study was to evaluate the neuroprotective potential of BF-7<sup>®</sup> in a mouse model of MPTP-induced PD. For experimental studies on PD, acute, subacute, or chronic administrations of MPTP in rodents is used to replicate pathological hallmarks shown in human PD patients (16,17). Thus, the present study assessed effects of BF-7<sup>®</sup> on motor behavior impairment, damage of dopaminergic neurons, DNA oxidation, lipid peroxidation and changes of antioxidant enzymes in a mouse model of MPTP-induced PD.

## Materials and methods

**Experimental animals.** A total of 30 adult male C57BL/6 mice (10 weeks old; 27±2 g) were used for this experiment. The mice were obtained from the Experimental Animal Center of Kangwon National University (Chuncheon, South Korea). They were housed in pathogen-free condition with standard temperature (~23°C) and humidity (~60%) on 12-h light/dark cycle. Freely accessible pellet feed (DBL Co., Ltd.) and water were provided to the animals.

The protocol of all experiments was approved on 28 January, 2020 by the Ethics Committee of Kangwon National University (approval no., KW-200113-2). All experimental procedures adhered to the guidelines described in the 'Current International Laws and Policies', which is included in the Guide for the Care and Use of Laboratory Animals (18). Every effort was made to reduce the pain of mice and minimize the number of mice used.

**Preparation of BF-7<sup>®</sup>.** BF-7<sup>®</sup> used in this experiment was supplied by Famenity Co., Ltd. BF-7<sup>®</sup> is a mixture of silk peptides obtained from the cocoon shell of silkworm (*Bombyx mori*). It was manufactured by a unique enzymatic hydrolysis process.

**Experimental groups and MPTP and BF-7<sup>®</sup> treatments.** A total of 30 mice were randomly assigned to three groups: i) Vehicle (saline) treated group (control group, n=10),

ii) vehicle and MPTP treated group (vehicle + MPTP group, n=10) and iii) BF-7<sup>®</sup> + MPTP group (n=10).

To produce experimental PD in mice, as previously described (17,19), 20 mg/kg MPTP (MilliporeSigma) dissolved in saline was intraperitoneally injected four times at an interval of two hours in one day (Fig. 1). For treatment with BF-7<sup>®</sup>, mice received 10 mg/kg BF-7<sup>®</sup> dissolved in saline and orally administered using a curved feeding needle (20-gauge; Kent Scientific Corporation) once a day for one week (from three days before MPTP treatment to three days after MPTP treatment; Fig. 1). At three days after MPTP treatment for analyses, all mice were deeply anesthetized by an intraperitoneal injection of pentobarbital sodium 200 mg/kg (JW Pharm. Co., Ltd.) and sacrificed.

**Rotarod test.** Rotarod test is a well-established way to assess motor coordination and balance for screening side effects of insults in preclinical tests (20). The present study used rotarod apparatus obtained from Ugo Basile North America Inc., which consisted of a horizontal rod (three cm in diameter) separated by plastic partitions to accommodate up to five mice per trial. In this test, the rotarod was set to accelerate from zero to 40 rpm in 300 sec. To acclimatize the mice to the equipment and the test, mice were trained for three consecutive days. On day -2, -1, 1 and 2 before and after MPTP treatment, the duration that each mouse remained on the rotarod was automatically recorded.

**Fluoro-Jade B (F-J B) staining.** F-J B was used as a fluorescent marker for degeneration of neurons. In this experiment, F-J B staining was used to detect loss/death of neurons in the substantia nigra. For the preparation of brain sections containing the substantia nigra, mice were deeply anesthetized with 200 mg/kg pentobarbital sodium obtained from JW pharm. Co., Ltd. and fixed transcardially with 4% paraformaldehyde. Obtained brains were cut into 20- $\mu$ m coronal sections using an SM2020 R sliding microtome (Leica Microsystems GmbH) equipped with a freezing stage (BFS-40MP; Physitemp Instruments Inc.). F-J B staining was performed according to a published method (21) with minor modification. Briefly, F-J B (MilliporeSigma) solution was prepared as 0.0003% F-J B in acetic acid. Sections were incubated with 0.06% potassium permanganate for 15 min at room temperature, washed with distilled water (DW) and incubated in the F-J B solution for 20 min at room temperature. After briefly washing, sections were warmed at ~50°C for the F-J B reaction completely dried. Thereafter, sections were cleared and coverslipped.

For the analysis for neuronal loss/death in the substantia nigra, seven sections per mouse were selected at corresponding levels. F-J B-stained cells were examined and images captured using a fluorescence microscope (Carl Zeiss AG) equipped with blue (450-490 nm) excitation light. F-J B-stained cells were counted in an area of 200  $\mu$ m<sup>2</sup> using an image analyzing software (Optimas 6.5; CyberMetrics Corporation).

**Immunohistochemistry.** Changes in dopaminergic neurons, lipid peroxidation, DNA oxidation and antioxidant proteins were examined by immunohistochemistry using rabbit anti-TH (diluted 1:200; Abcam; cat. no. ab6211), mouse anti-4HNE (diluted 1:1,200; Abcam; cat. no. ab48506), goat anti-8OHdG (diluted 1:600; MilliporeSigma; cat. no. AB5830), sheep anti-SOD1 (diluted 1:500; Calbiochem; cat. no. 574597) and sheep anti-SOD2

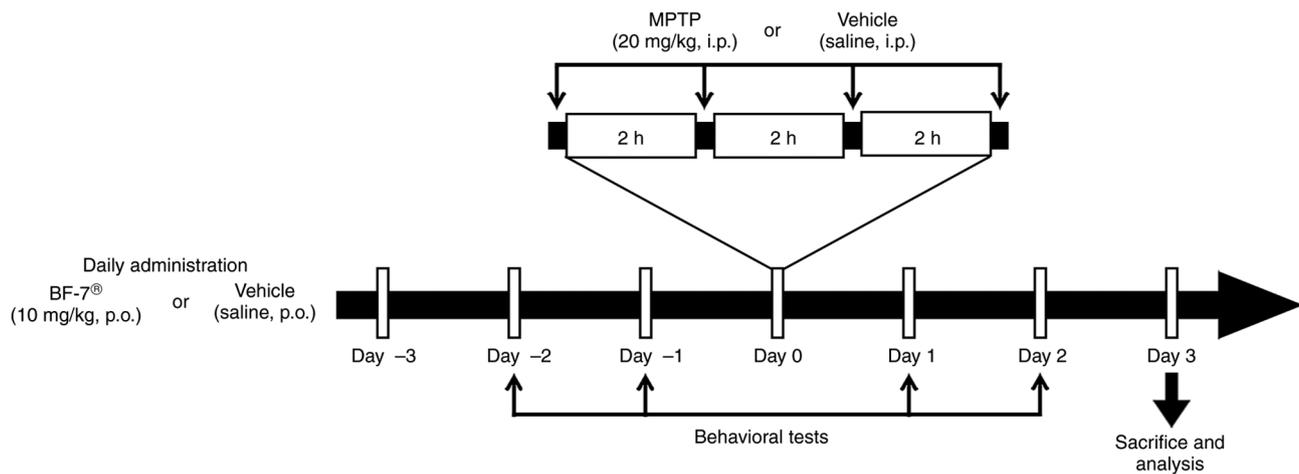


Figure 1. Experimental timeline. At day 0, MPTP (20 mg/kg) and vehicle was intraperitoneally injected four times with two hours of interval. BF-7<sup>®</sup> and vehicle (saline) was administrated once a day for seven days. Behavioral test was performed at one and two days before MPTP treatment and one and two days after MPTP treatment. At three days after MPTP treatment, mice were sacrificed for analyses. MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; BF-7<sup>®</sup>, Brain Factor-7<sup>®</sup>.

(diluted 1:1,000; Calbiochem; cat. no. 574596). According to a published method (22) with some modifications, sections (described above) were immersed in 0.3% H<sub>2</sub>O<sub>2</sub> for 25 min at room temperature (RT) to block endogenous peroxidase activity. Sections were then incubated with 5% goat (cat. no. S-1000-20), rabbit (cat. no. S-5000-20) or horse (cat. no. S-2000-20) serum (Vector Laboratories Inc.) for 25 min at RT to block non-specific immunoreaction. Thereafter, sections were immunoreacted with the primary antibodies for 12 h at 4°C and exposed to biotinylated goat anti-rabbit IgG (cat. no. BA-1000-1.5), horse anti-mouse IgG (cat. no. BA-2001-1.5), rabbit anti-goat IgG (cat. no. BA-5000-1.5) or rabbit anti-sheep IgG (cat. no. BA-6000-1.5) (diluted 1:250; Vector Laboratories Inc.) and streptavidin peroxidase complex (diluted 1:250; Vector Laboratories Inc.). The immunoreaction in sections was visualized with 3,3'-diaminobenzidine tetrahydrochloride (0.06% DAB agar; MilliporeSigma) and 30% H<sub>2</sub>O<sub>2</sub> for one min at RT. Finally, sections were washed, dehydrated, cleared and coverslipped.

To analyze numbers of TH-immunostained cells, seven sections per mouse were selected at corresponding levels of the substantia nigra. Images of the TH-immunostained cells were captured using a BX53 upright light microscope (Olympus Corporation). Then two blinded experimenters counted mean numbers of TH-immunostained cells at corresponding areas using an image analyzing system (Optimas 6.5; CyberMetrics Corporation).

To analyze immunoreactivity of SOD1, SOD2, 4HNE and 8OHdG, the choice of the sections and the capture of the images were performed in the same way as described above. Change in each immunoreactivity was presented as a relative optical density (ROD) using Adobe Photoshop 8.0 (Adobe Systems, Inc.) and ImageJ software 1.59 (National Institutes of Health). ROD was calibrated as % compared with the control group (100%).

**Statistical analysis.** Regarding sample size, ≤7 mice per group were used at an  $\alpha$  error of 0.05 and a power of >80%. The sample size was calculated with power calculator. Using GraphPad Prism software (version 5.0; GraphPad Software, Inc.), a multiple-sample comparison was performed to test the

differences between the groups (two-way analysis of variance and Tukey's multiple comparison with a *post hoc* test using the criterion of the least significant differences). All data were presented as mean ± standard error of mean (SEM). P<0.05 was considered to indicate a statistically significant difference.

## Results

**BF-7<sup>®</sup> alleviated MPTP-induced motor deficit.** In the MPTP-induced mice, motor deficit was evaluated to investigate the relationship between DA neuron degeneration using rotarod test and the effect of BF-7<sup>®</sup> administration in the MPTP-induced mice was assessed (Fig. 2). Rotarod analysis exhibited significant movement deficit in the vehicle + MPTP group as the mice were not able to perform on the rotarod for longer time: they felt down significantly earlier as compared with the control group. Rotarod analysis in the mice treated with BF-7<sup>®</sup> showed that the latency (39.8 sec at one day after MPTP treatment and 60.5 sec at two days after MPTP treatment) was significantly longer compared with that in the vehicle + MPTP group (22 sec at one day after MPTP treatment and 33.6 sec at two days after MPTP treatment). This finding suggested that motor deficit induced by MPTP treatment was alleviated by BF-7<sup>®</sup> administration.

**BF-7<sup>®</sup> protects dopaminergic neurons from MPTP-induced cell death.** After noting that BF-7<sup>®</sup> administration alleviated MPTP-induced motor deficit, the present study continued to examine if there were neuroprotective effects of BF-7<sup>®</sup> against MPTP-induced dopaminergic cell loss using TH immunohistochemistry and/or F-J B staining in the substantia nigra.

**Findings in SNpc.** Treatment with 10 mg/kg BF-7<sup>®</sup> protected dopaminergic neurons in the SNpc from MPTP-induced neuronal death.

In the control group, TH-stained dopaminergic cells (neurons) were distributed in SNpc (Fig. 3Aa, Ad and B) and no F-J B-stained cells (degenerating cells) were found in the SNpc (Fig. 3Ag and C). In the vehicle + MPTP group,

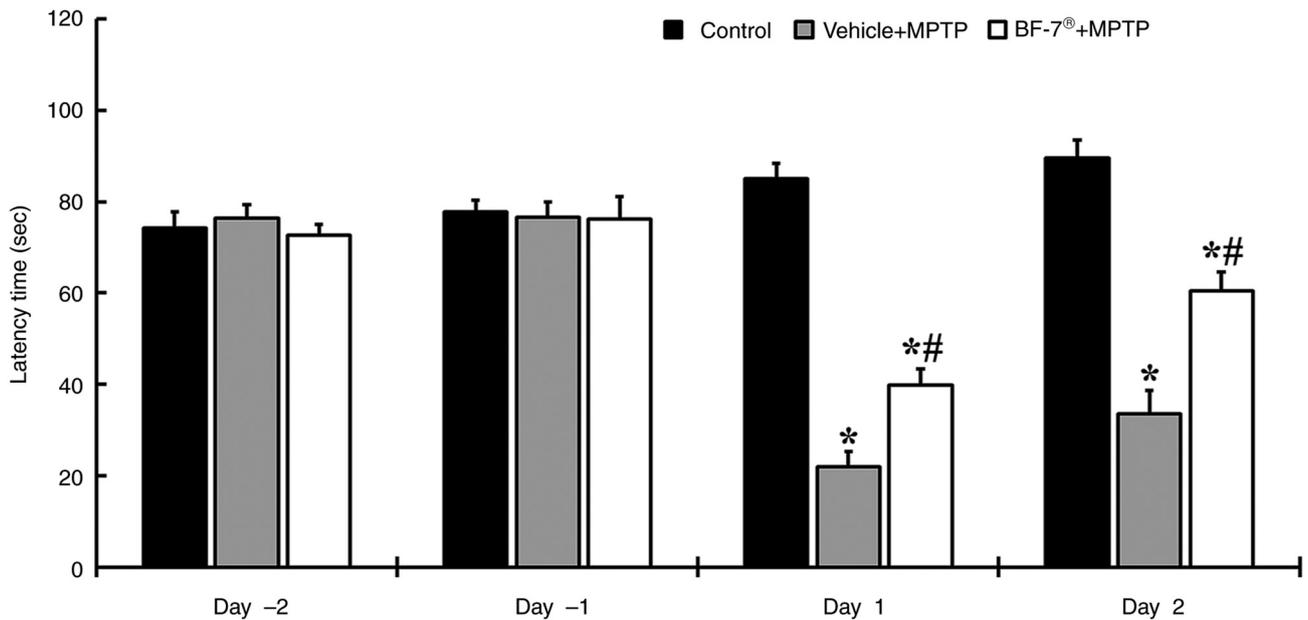


Figure 2. Effects of BF-7<sup>®</sup> on MPTP-induced motor impairment in mice. Latency time on the rotarod is impaired in the vehicle + MPTP group from day 1 to 2 following MPTP treatment. However, the impairment is ameliorated in the BF-7<sup>®</sup> + MPTP group (n=10, respectively; \*P<0.05 vs. control group and #P<0.05 vs. vehicle + MPTP group). The bars indicate the means  $\pm$  standard error of mean. BF-7<sup>®</sup>, Brain Factor-7<sup>®</sup>; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

TH-stained dopaminergic cells were significantly reduced (37.3% of control; Fig. 3Ab, Ae and B) and numerous F-J B-stained cells (54.7 cells/200  $\mu\text{m}^2$ ) cells were shown three days after MPTP administration (Figs. 3Ah and C). However, in the BF-7<sup>®</sup> + MPTP group, TH-stained dopaminergic cells were significantly protected (82.7% of control group; Fig. 3Ac, Af and B) and F-J B-stained cells were significantly reduced (23.4% of vehicle + MPTP group; Fig. 3Ai and C).

*Findings in striatum.* Treatment with 10 mg/kg BF-7<sup>®</sup> ameliorated MPTP-induced decrease of TH immunoreactivity in the striatum.

The striatum receives dopamine from dopaminergic cells located in the SNpc. In the control group, strong TH immunoreactivity, which is dopaminergic neuropil, was shown in the striatum (Figs. 3B and 4Aa). In the vehicle + MPTP group, TH immunoreactivity was significantly reduced (12.8% of control) compared with the control group three days after MPTP treatment (Fig. 4Ab and B). However, in the BF-7<sup>®</sup> + MPTP group, TH immunoreactivity in the striatum was very high (89.8% of control) three days after MPTP treatment (Figs. 4Ac and B).

*BF-7<sup>®</sup> alleviates MPTP-induced lipid peroxidation and DNA oxidation.* Treatment with 10 mg/kg BF-7<sup>®</sup> reduced MPTP-induced oxidative stresses in the SNpc.

*4HNE immunoreactivity.* The immunoreactivity of 4HNE (a marker for lipid peroxidation) in the control group was very weak in the SNpc (Fig. 5Aa). In the vehicle + MPTP group, 4HNE immunoreactivity was apparently enhanced (288.7% of control) in the SNpc three days after MPTP administration compared with the control group (Fig. 5Ab and B). However, in the BF-7<sup>®</sup> + MPTP group, 4HNE immunoreactivity in the SNpc was significantly reduced (51.5% of vehicle + MPTP group) compared with the vehicle + MPTP group (Fig. 5Ac and B).

*8OHdG immunoreactivity.* In the control group, 8OHdG immunoreactivity was observed in the SNpc (Fig. 5Ad and C). In the vehicle + MPTP group, 8OHdG immunoreactivity in the SNpc was significantly enhanced (222.9% of control) three days after MPTP administration (Fig. 5Ae and C). However, in the BF-7<sup>®</sup> + MPTP groups, the 8OHdG immunoreactivity was significantly decreased (68.7% of vehicle + MPTP group) compared with the vehicle + MPTP group (Fig. 5Af and C).

*BF-7<sup>®</sup> alleviates MPTP-induced decrease in antioxidant enzymes.* Treatment with 10 mg/kg BF-7<sup>®</sup> ameliorated MPTP-induced decrease of endogenous antioxidant enzymes.

*SOD1 immunoreactivity.* SOD1 immunoreactivity was shown in the SNpc of the control group (Fig. 6Aa and B). In the vehicle + MPTP group, SOD1 immunoreactivity in the SNpc was significantly decreased (32.0% of control) three days after MPTP administration (Fig. 6Ab, B). However, in the BF-7<sup>®</sup> + MPTP group, SOD1 immunoreactivity in the SNpc was significantly increased (220.8% of vehicle + MPTP group) as compared with the vehicle + MPTP group (Fig. 6Ac, B).

*SOD2 immunoreactivity.* In the control group, SOD2 immunoreactivity was also found in the SNpc (Fig. 6Ad and C). In the vehicle + MPTP group, SOD2 immunoreactivity in the SNpc was significantly decreased (44.4% of control) three days after MPTP administration (Fig. 6Ae and C). However, SOD2 immunoreactivity in the BF-7<sup>®</sup> + MPTP group was significantly increased (176.1% of vehicle + MPTP group) as compared with the vehicle + MPTP group (Fig. 6Af and C).

## Discussion

PD is a neurodegenerative disease accompanied by gradual loss of dopaminergic neurons in SNpc of the midbrain (2). These

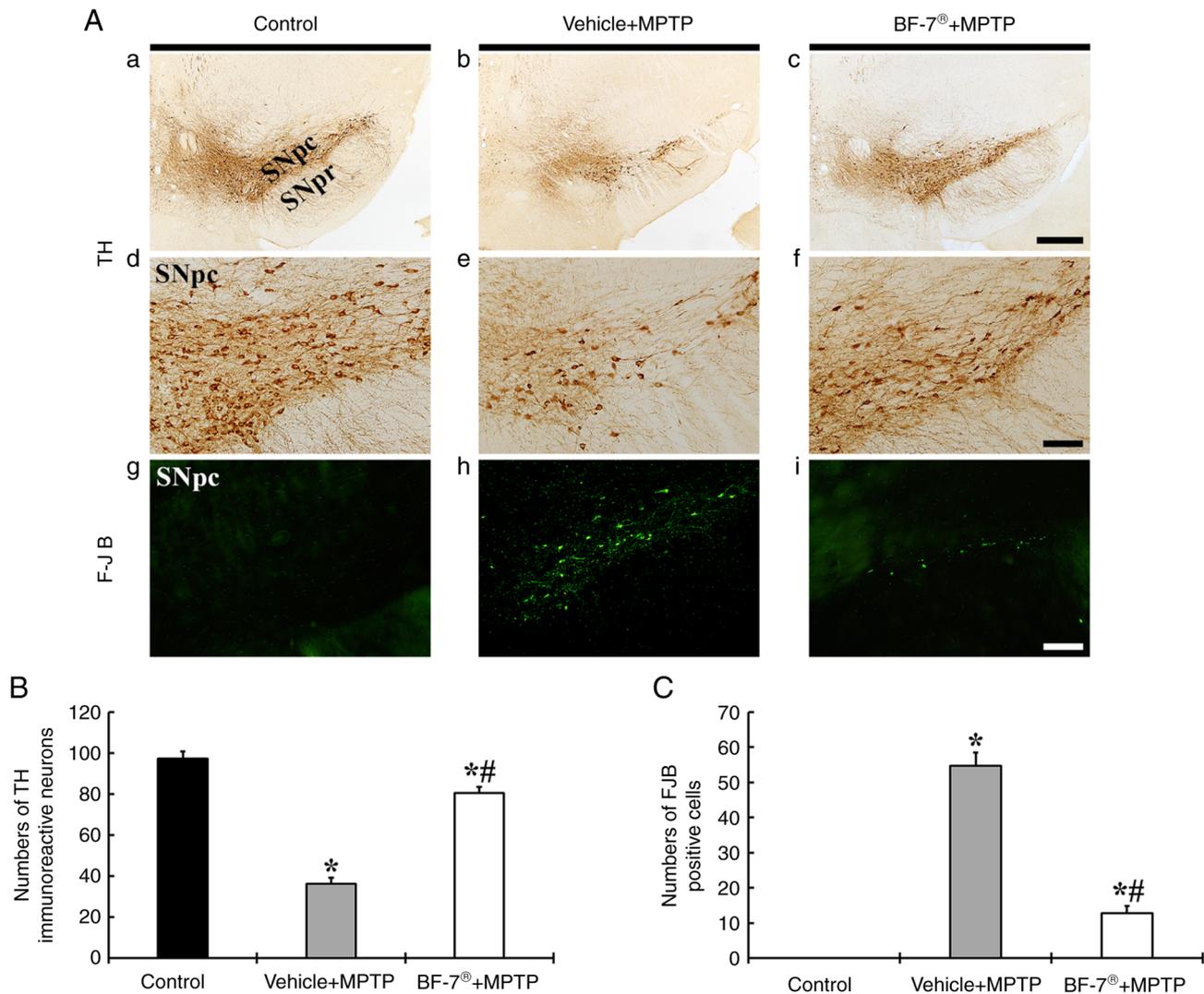


Figure 3. Immunohistochemistry for TH and F-J B histochemistry in the SNpc. Representative images of (Aa-Af) TH immunohistochemistry and (Ag-Ai) F-J B staining in the SNpc of the (a, d and g) control group, (b, e and h) vehicle + MPTP group and (c, f and i) BF-7<sup>®</sup> + MPTP group three days after MPTP treatment. In the vehicle + MPTP group, TH-stained dopaminergic cells are significantly reduced compared with the control group and F-J B-stained cells are abundantly found; however, in the BF-7<sup>®</sup> + MPTP group, the number of TH-stained dopaminergic cells is high as compared with the vehicle + MPTP group and the number of F-J B-stained cells is significantly low as compared with the vehicle + MPTP group. Scale bars (a-c) 400 and (d-i) 50  $\mu$ m. Mean number of (B) TH-stained and (C) F-J B-stained cells (n=10, respectively; \*P<0.05 vs. control and #P<0.05 vs. vehicle + MPTP group). The bars indicate the means  $\pm$  standard error of mean. TH, tyrosine hydroxylase; F-J B, Fluoro-Jade B; SNpr, substantia nigra pars reticulata.

neurons can secrete dopamine, an organic chemical of the catecholamine and phenethylamine families that serves crucial roles in regulating the relaxation and balance of movements (2). Motor symptoms of PD include tremor, rigidity, slowness of movement, falls and dizziness, freezing, muscle cramps and dystonia (2). In addition, PD displays olfaction deficit, rapid eye movement sleep disorders, depression, constipation and cognitive impairment by affecting cholinergic, serotonergic and noradrenergic systems (2). These symptoms are caused by selective and progressive degeneration or loss of dopaminergic neurons in the SNpc and depletion of dopaminergic nerve fibers projecting to the striatum from the SNpc (2).

It has been reported that MPTP treatment to mice for PD model can result in significant movement disorders, including increased climbing time and reduced swimming time (23-25). In addition, motor dysfunctions in MPTP-treated mice can be evaluated by rotarod performance and open-field tests (26).

The authors previously reported that treatment with 10 mg/kg BF-7<sup>®</sup> showed neuroprotective effects in a gerbil model of transient forebrain ischemia and in a rat model of transient focal cerebral ischemia (15). Based on the previous findings, the dose of BF-7<sup>®</sup> in the present study was selected as 10 mg/kg. In the current study, rotarod analysis exhibited significant movement deficit mice of the vehicle + MPTP group. Therefore, the effects of BF-7<sup>®</sup> on motor deficit in MPTP-treated mice as investigated. It was found that the motor deficit of the mice with PD was significantly improved by BF-7<sup>®</sup> treatment. This finding was consistent with previous results showing that metformin or gastrodin could improve motor deficits in MPTP-induced Parkinson's disease in mice (27,28).

In clinical reports, motor symptoms in PD patients are firstly shown at 50-60% loss (death) of dopaminergic cells in the SNpc and 70-80% degradation of dopamine level in the striatum (29,30). In the present study, BF-7<sup>®</sup> administration

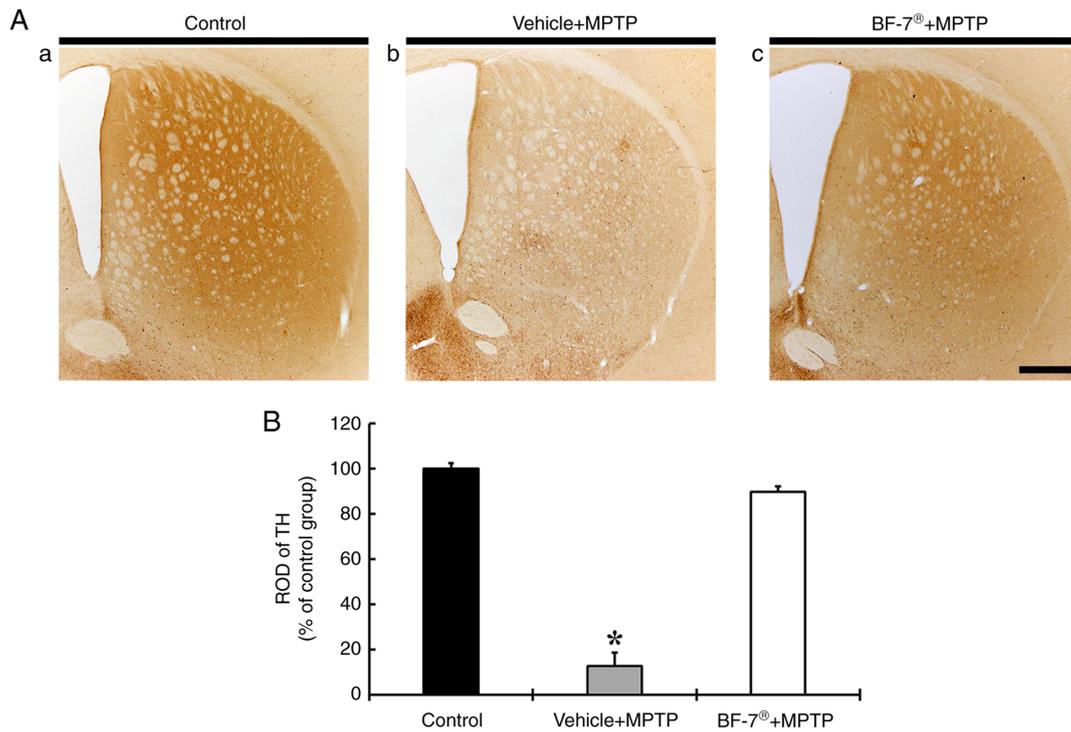


Figure 4. Immunohistochemistry for TH in the striatum. (A) TH immunoreactivity in the striatum of the (a) control group, (b) vehicle + MPTP group and (c) BF-7<sup>®</sup> + MPTP group three days after MPTP treatment. TH immunoreactivity in the control group is strong. In the vehicle + MPTP group, TH immunoreactivity is significantly decreased three days after MPTP treatment; however, the TH immunoreactivity is ameliorated in the BF-7<sup>®</sup> + MPTP group compared with the vehicle + MPTP group. Scale bar=200  $\mu$ m. (B) ROD of TH immunoreactivity in the striatum (n=10, respectively; \*P<0.05 vs. control group). The bars indicate the means  $\pm$  standard error of mean. TH, tyrosine hydroxylase; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; BF-7<sup>®</sup>, Brain Factor-7<sup>®</sup>; ROD, relative optical density.

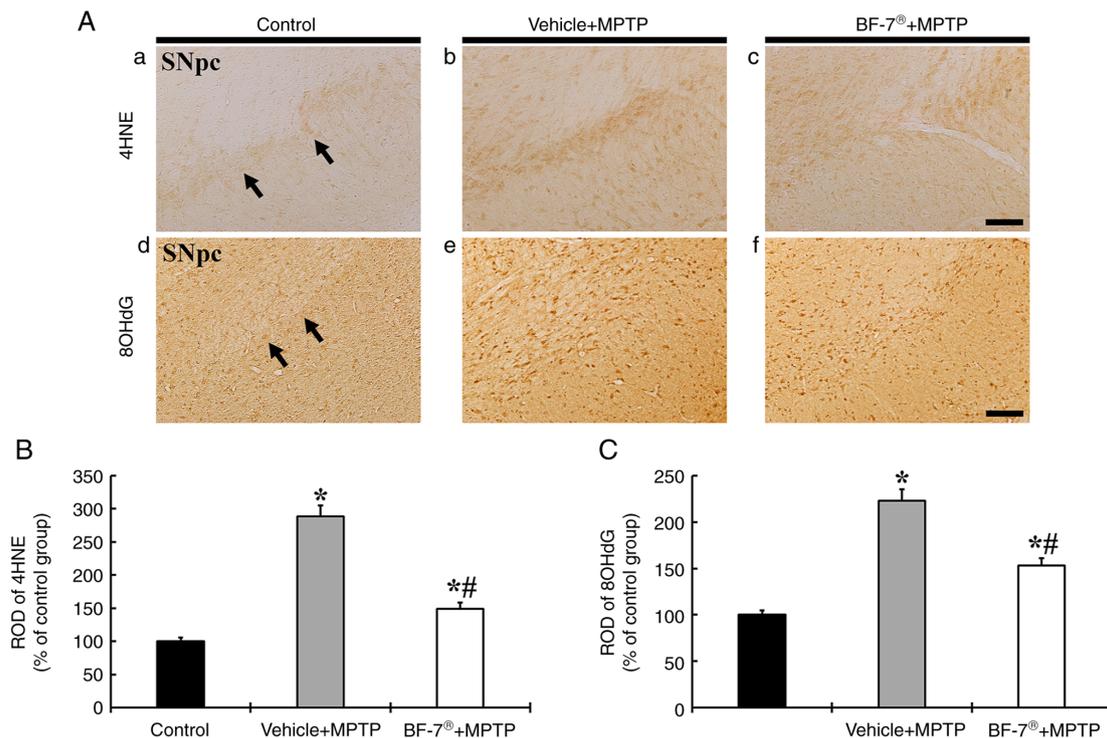


Figure 5. Immunohistochemistry for 4HNE and 8OHdG in the SNpc. Immunohistochemical images of (Aa-Ac) 4HNE and (Ad-Af) 8OHdG in the SNpc of the (a and d) control group, (b and e) vehicle + MPTP group and (c and f) BF-7<sup>®</sup> + MPTP group three days after MPTP treatment. 4HNE and 8OHdG immunoreactivity (arrows) are shown in the SNpc of the control group. In the vehicle + MPTP group, 4HNE and 8OHdG immunoreactivity are increased three days following MPTP treatment; however, the two increased immunoreactivities are apparently reduced in the BF-7<sup>®</sup> + MPTP group as compared with the vehicle + MPTP group. Scale bar=50  $\mu$ m. ROD of (B) 4HNE and (C) 8OHdG immunoreactivity in the SNpc three days following MPTP treatment (n=10, respectively; \*P<0.05 vs. control group and #P<0.05 vs. vehicle + MPTP group). The bars indicate the means  $\pm$  standard error of mean. 4HNE, 4-hydroxy-2-nonenal; 8OHdG, 8-hydroxydeoxyguanosine; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; SNpc, pars compacta of substantia nigra; ROD, relative optical density.

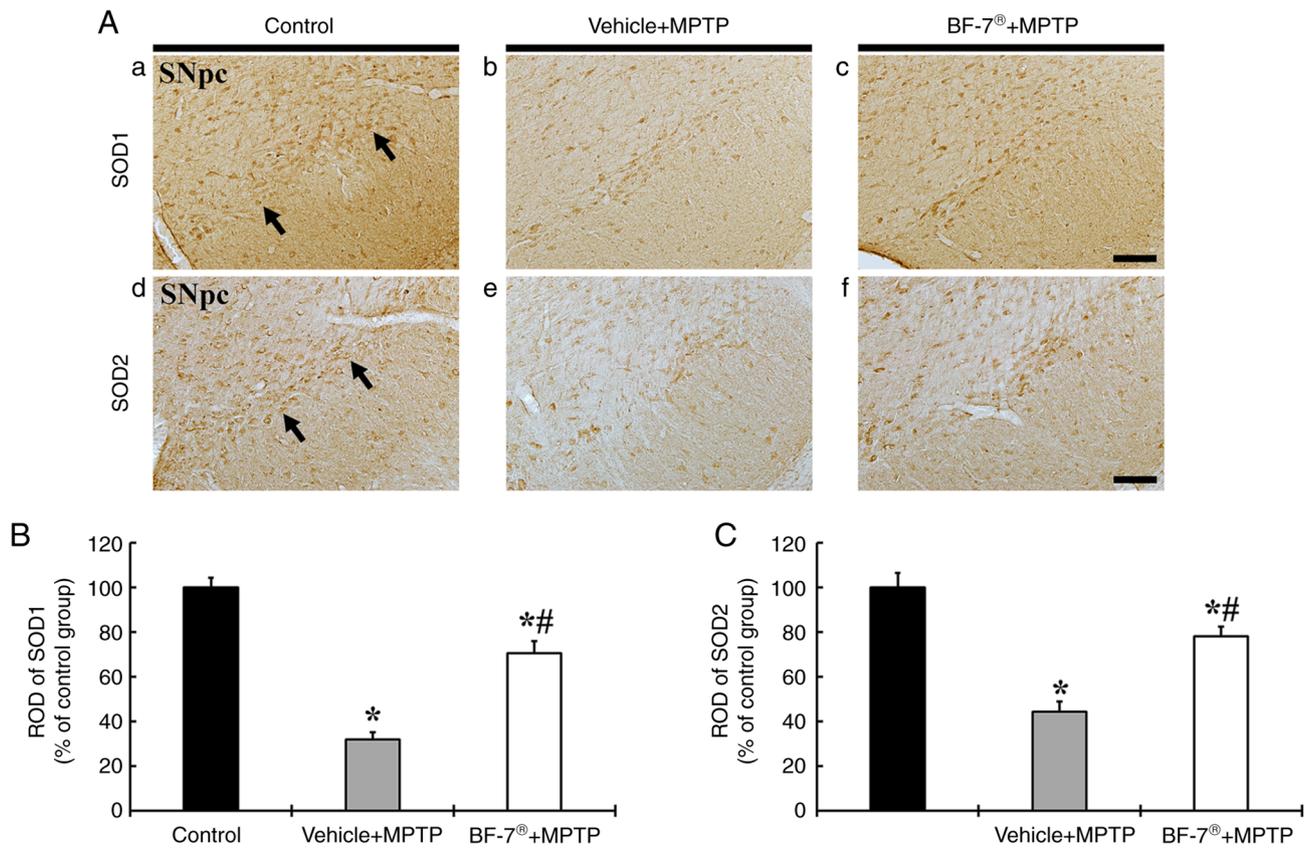


Figure 6. Immunohistochemistry for SOD1 and SOD2 in the SNpc. Representative images of (Aa-Ac) SOD1 and (Ad-Af) SOD2 immunoreactivity in the SNpc of the (a and d) control group, (b and e) vehicle + MPTP group and (c and f) BF-7<sup>®</sup> + MPTP group three days after MPTP treatment. SOD1 and SOD2 immunoreactivity (arrows) are shown in the SNpc of the control group. In the vehicle + MPTP group, SOD1 and SOD2 immunoreactivity are apparently decreased three days after MPTP treatment. However, the two decreased immunoreactivities are higher in the BF-7<sup>®</sup> + MPTP group than the vehicle + MPTP group. Scale bar=50  $\mu$ m. Scale bar=50  $\mu$ m. ROD of (B) SOD1 and (C) SOD2 immunoreactivities in the SNpc three days following MPTP treatment (n=10, respectively; \*P<0.05 vs. control group and #P<0.05 vs. vehicle + MPTP group). The bars indicate the means  $\pm$  standard error of mean. SOD, superoxide dismutase; SNpc, pars compacta of substantia nigra; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; ROD, relative optical density.

improved the motor deficit in the vehicle + MPTP group. Thus, whether BF-7<sup>®</sup> could protect dopaminergic cells from MPTP-induced injury in mice was examined using immunohistochemistry for TH, a rate-limiting enzyme for dopamine synthesis that could be used as a marker of dopaminergic cell loss in the SNpc of a MPTP-intoxicated PD mouse model (31). MPTP administration mainly causes a decrease in TH density in the nigrostriatal pathway, which conveys dopamine from the SNpc to the striatum, leading to a loss of projecting striatal dopaminergic nerve terminals in mice (30). The immunohistochemical analysis in the present study showed that administration with MPTP significantly decreased numbers of TH-immunoreactive dopaminergic cells in the SNpc and TH immunoreactivity in the striatum compared with the control, consistent with previous reports (32-34). Additionally, in the BF-7<sup>®</sup> + MPTP group, BF-7<sup>®</sup> rescued the debilitating effects of MPTP. It protected dopaminergic neurons against MPTP-induced neurodegeneration. Improved dopaminergic cells in the SNpc and dopamine level in the striatum corroborated with the alleviation of motor deficit by BF-7<sup>®</sup> treatment in a mouse model of MPTP-induced PD.

Lipids, as major components of the central nervous system serve crucial roles in neural health and pathology (35). Disturbance of lipid metabolism, particularly lipid peroxidation, is associated with the development of many neurodegenerative

diseases, including PD and Alzheimer's disease, in which levels of lipid peroxidation products and lipid peroxidation-modified proteins are elevated (36). Therefore, inhibition of neuronal oxidation can delay or reduce the progression and severity of neurodegenerative diseases (36). HNE is a product of lipid peroxidation that contributes to apoptotic cell death via caspase cascade activation and subsequent induction of DNA fragmentation (37). The present study found that MPTP-induced increase of 4HNE immunoreactivity in dopaminergic cells of the SNpc were significantly decreased by treatment with BF-7<sup>®</sup>.

DNA integrity is a prerequisite for cell survival (38). Under pathological conditions, DNA can be damaged by endogenous and/or environmental toxic agents. DNA damage is known to contribute to genetic and protein instability and subsequent cell death (38). Dopaminergic cells are commonly exposed to ROS assault. DNA oxidative damage happens when ROS production is excessive (39). In the current study, 8OHdG (a marker of DNA oxidative damage) immunoreactivity was significantly increased in the SNpc of the vehicle + MPTP group. However, 8OHdG immunoreactivity in the BF-7<sup>®</sup> + MPTP group was significantly lower compared with that in the vehicle + MPTP group. 8OHdG concentration is selectively elevated in the SNpc of PD patients (40). Furthermore, PD patients consistently show higher 8OHdG levels in cerebrospinal fluid compared with normal controls (41,42). In oxidative stress, one

of DNA lesions induced by ROS is an oxidized form of 8OHdG as a biomarker of DNA damage (43). It has been reported that MPTP insults can generate ROS including superoxide anions. The interaction between ROS and DNA causes DNA strand break and base modification, which can be assessed by measuring the level of nucleoside 8OHdG (44).

Dopaminergic neurons in PD are thought to be affected by high oxidative stress from enzymatic and non-enzymatic metabolism and dopamine autoxidation (45,46). SOD1 (a copper/zinc protein) and SOD2 (a mitochondrial manganese enzyme), as enzymatic antioxidants can metabolize superoxide radicals to molecular oxygen and H<sub>2</sub>O<sub>2</sub> or scavenge oxygen radicals produced by electron-transport reactions and oxidation-reduction in mitochondria (47,48). Notably, it has been reported that SOD1 and SOD2 transgenic mice are resistant to dopaminergic neurotoxin MPTP (49,50). In addition, increased SOD2 in a rat model of PD show an increase in survival of transplanted cells from 6-OHDA-induced neurotoxicity (51). Furthermore, overexpression of SOD1 in a *Drosophila* model of PD can protect against dopaminergic neuronal loss induced by mutant  $\alpha$ -synuclein (52). The current study also showed that SOD1 and SOD2 expression levels were enhanced in the BF-7<sup>®</sup> + MPTP group compared with the vehicle + MPTP group.

The present study examined the neuroprotective effect of BF-7<sup>®</sup> in a mouse model of Parkinson's disease. However, the current study has a limitation in not investigating the pathways that inhibit and/or alleviate oxidative stresses. Therefore, it is proposed that a follow-up study using *in vitro* approach needs to be conducted for in-depth investigation of the neuroprotective effect of BF-7<sup>®</sup>. In further studies, changes in levels of neurotransmitters including dopamine, 3,4-dihydroxyphenylacetic acid and homovanillic acid should be investigated to examine how BF-7<sup>®</sup> affects behavioral index in a mouse model of Parkinson's disease.

In conclusion, the present study provided evidence that BF-7<sup>®</sup> can exert a neuroprotective effect against MPTP-induced PD in mice, showing that BF-7<sup>®</sup> treatment can improve motor deficit, alleviate loss or decrease in dopaminergic cells and nerve terminals and reduce oxidative stress (such as lipid peroxidation, DNA damage and decrease in SODs) in a mouse model of MPTP-induced PD. Therefore, the results strongly suggested that BF-7<sup>®</sup> has potential as a candidate for treating PD.

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### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

TKL, JCL, DWK, JWJ, HIK and MCS performed the experiments and measurements. TKL, JCL, SSK and JHC analyzed and interpreted data. TKL, JCL and MHW confirm the authenticity of all the raw data. and MHW and SYC made substantial contributions to conception and design and were involved in drafting, revising the manuscript and interpreting all data. All authors have read and approved for the final manuscript.

### Ethics approval and consent to participate

The protocol of all experiments was approved on January 28, 2020 by the Ethics Committee of Kangwon National University (approval no. KW-200113-2). All experimental procedures adhered to the guidelines described in the 'Current International Laws and Policies', which is included in the Guide for the Care and Use of Laboratory Animals. Every effort was made to reduce the pain of mice and minimize the number of mice used.

### Patient consent for publication

Not applicable.

### Competing interests

The authors have declared that there is no conflicting interest. Note that J-WL is employed by Famenity Co., Ltd., who produced the drug BF-7<sup>®</sup> used in this study.

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