Original Article

Ginseng extract G115 improves locomotor function in rotenone-induced parkinsonism rats via an antioxidant effect

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Abstract

Exposure to certain pesticides including rotenone, a mitochondrial complex I inhibitors, may cause the oxidative damage to the dopaminergic neurons and contribute to Parkinson's disease. Herein we demonstrate that ginseng extract G115 could attenuate locomotor activity impairment in rotenone-induced parkinsonism rats. Rotenone was shown to significantly impair the movement of rat related to its ability to reduce free radical scavenging capability and superoxide dismutase (SOD) activity in the rat brain. Ginseng extract G115 (400 mg/kg) given 14 days before and concurrently with rotenone significantly protected against rotenone-induced locomotor impairment at day 17, but not day 20. In addition, the treatment with ginseng extract G115 (400 and 800 mg/kg) significantly prevented the loss of free radical scavenging and SOD activities in rotenone-treated rats while inhibiting the lipid peroxidation. Together, the present study provides mechanistic insight into rotenone-mediated PD as well as the information of ginseng extract that is promising for the prevention.

Keywords: ginseng extract G115, rotenone, parkinsonism, antioxidant effect

1. Introduction

Parkinson's disease (PD) is an important complex neurodegenerative age-related disorder (De Lau and Breteler, 2006). The disease is characterized by an irreversible loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc), resulting in a defect of smooth motor ability (Dauer and Przedborski, 2003). Although the exact mechanism of PD pathogenesis is still unknown, the excessive production of reactive oxygen species (ROS) has been shown to have a significant impact on the pathogenesis as well as the progression of the disease (Tieu *et al.*, 2003). The impairment of mitochondrial complex I function in the SNpc is a predominant factor conferring ROS-dependent neural apoptosis in the PD patients (Kean *et al.*, 2011; Parker *et al.*, 2008; Perier and Vila, 2012). Neurons are among the cells most sensitive to oxidative impairment in comparison to the cell components of other tissue. Accumulation or aggravation of intracellular ROS in neurons caused by many means, including endoplasmic reticulum (ER) stress, lysosomal membrane disruption, and mitochondrial dysfunction, induces apoptosis of the cells through intrinsic death mechanism (Annunziato *et al.*, 2003; Lin *et al.*, 2012). Though the precise mechanism mediating increased ROS level in the neurons of PD patients is still largely unknown, it is widely accepted that the oxidative stress plays a critical part in the generation and the progression of the disease.

In accordance with the aforementioned concept, compounds possessing anti-oxidant activity are among the potential targets of research for their promising merit to be used in PD patients. Ginseng extracts and ginsenosides have been tested in various neurodegenerative diseases models

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in vitro and *in vivo*. Ginsenosides Rg1 and Rb1 increased neurite outgrowth in PC-12 cells and reversed MPTP-induced SK-N-SH cells death (Rudakewich *et al.*, 2001). In addition, ginsenosides Rg1 and Rb1 were shown to increase the survival of dopaminergic cells against MPP$^+$ neurotoxicity in mesencephalic culture (Radad *et al.*, 2004). *In vivo* study revealed that prolonged oral administration of ginseng extract G115 protected against MPP$^+$-induced reductions of tyrosine hydroxylase-positive neurons in the SNpc of rats and MPTP-induced neurotoxicity in mice (Van Kampen *et al.*, 2003). Ginseng extract G115 is a well-established standardized extract of *Panax ginseng* which contains 4% ginsenosides. Studies using high performance liquid chromatography (HPLC) reported that ginseng extract G115 is composed of ginsenosides Rb1, Rb2, Rc, Rd, Re, Rg1 (Leung and Wong, 2010). So far, the effects of ginseng extract G115 on the protection of rotenone-induced parkinsonism rats have been largely unknown, especially in the relation of oxidative damage. Therefore, the present study aimed to determine the neuroprotective effect of ginseng extract G115 in rotenone-induced parkinsonism rats in behavioral parameters and the possible underlying mechanisms.

2. Materials and Methods

2.1 Animals

Male Wistar rats, weighing 300-350 g, (National Laboratory Animal Center, Mahidol University, Nakornpathom, Thailand) were housed in groups of 3 per cage at a constant ambient temperature (25±2°C) and humidity (50-60%) under a 12-hour light/dark cycle with free access to food and water. All procedures were approved by the Institutional Animal Care and Use Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand (Approval No.13-33-010).

2.2 Chemicals

Rotenone, apomorphine, dimethyl sulfoxide (DMSO), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), horseradish peroxidase, thiobarbituric acid (TBA), 6-hydroxy-2,5,7,8,-tetramethylchroman-2-carboxylic acid (Trolox), sodium dodecyl sulfate (SDS), 1,1,3,3-tetraethoxy-propane (TEP), n-butanol, ethylenediamine tetraacetic acid (EDTA), nitroblue tetrazolium (NBT), bovine serum albumin (BSA), xanthine oxidase and copper (II) chloride were purchased from Sigma-Aldrich (St. Louis, MO, USA.). Gracial acetic acid and pyridine were purchased from BDH Laboratory Supplies (Dorset, UK). Sodium carbonate was purchased from Fisher Scientific UK Limited (Leicestershire, UK). Ginseng extract G115 (Ginsana® SA, Switzerland) was contained in soft gelatin capsules as 100 mg of ginseng extract G115 per capsule.

2.3 Chemical preparation

2.3.1 Ginseng extract G115

As Ginseng extract G115 (Ginsana® SA, Switzerland) was contained in capsules, each of 100 mg, 2, 4 and 8 capsules of ginseng extract G115 were dissolved in 1-mL of sterile water to make ginseng extract G115 in the concentration of 200, 400 and 800 mg/mL, respectively. Ginseng extract G115 was given orally to rats in a constant volume of 1 mL/kg body weight. Control group received sterile water (1 mL/kg) orally.

2.3.2 Rotenone

Rotenone was dissolved in 100% DMSO to prepare a stock solution in the concentration of 150 mg/mL. Working solution of rotenone (3 mg/mL) was freshly prepared by diluting in sunflower oil. The concentration of DMSO in the working solution was 2%. All rats in groups requiring rotenone were intraperitoneally injected with rotenone working solution in a constant volume of 1 mL/kg body weight while rats in the control group were injected with 2% DMSO in sunflower oil (1 mL/kg i.p.).

2.3.3 Apomorphine

Apomorphine was dissolved in sterile water in the concentration of 1 mg/mL, and given subcutaneously (s.c.) to all rats in constant volume of 1 mL/kg body weight.

2.4 Experimental design

Rats were randomly assigned to 5 groups (n = 12/group at the beginning of the experiment). On days 1-14, rats in control and PD group received sterile water (1 mL/kg p.o.) while rats in PD-G200, PD-G400 and PD-G800 were given ginseng extract G115 200, 400 and 800 mg/kg p.o., respectively. On days 15-20, rats in the control group were given sterile water (1 mL/kg p.o.) followed by 2% DMSO in sunflower oil (1 mL/kg i.p.) 30 minutes later while rats in PD group received sterile water (1 mL/kg p.o.) followed by rotenone (3 mg/kg i.p.) 30 minutes later to induce parkinsonism symptoms. Rats in PD-G200, PD-G400 and PD-G800 were given ginseng extract G115 200, 400 and 800 mg/kg p.o., respectively, followed by rotenone (3 mg/kg i.p.) 30 minutes later (Figure 1).

2.5 Behavioral testing

Locomotor activity was tested on the experimental day 1 (pretreatment), 14, 17 (after 3 days of rotenone treatment) and 20 (after 6 days of rotenone treatment). On the experimental day 1, locomotor activity was tested 1 hour
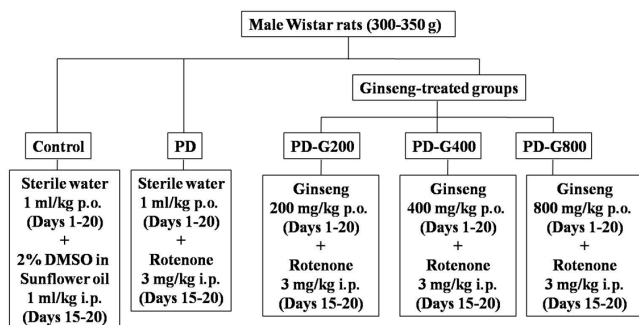
Figure 1. Schematic diagram of each experimental group.

before treatment while on the experimental day 14, 17 and 20, rats performed locomotor activity 30 minutes after treatment. A black square box (50 x 50 x 41 cm) was used for locomotor activity testing. Each rat was placed in the box for 5 minutes in the dark condition. VideoMOT2 system (TSE systems, Germany), a real time video recorder and analyzer, was used for tracking rats' locomotor activity.

2.6 Apomorphine challenge

To determine whether the observed behavioral deficits were dopamine-dependent, the ergot derivative dopamine agonist, apomorphine (1 mg/kg), was subcutaneously injected to all rats on day 21. Locomotor activity test was performed 5 minutes after apomorphine injection.

2.7 Biochemical analysis

To determine the neuroprotective mechanism of ginseng extract G115, all rats were euthanized by pento-barbital sodium (200 mg/kg i.p.) on the experimental day 22. Brain was quickly removed and washed in 0.1 M phosphate buffer saline (PBS), and kept frozen at -80°C for later analysis of free radical scavenging capacity, malondialdehyde (MDA) level and superoxide dismutase (SOD) activity. 0.1 M PBS (pH 7.4) at 4°C was added 1:10 w/v to the rat brain and, then homogenized using a hand homogenizer (Glas-Col®, USA). Brain homogenate was then transferred into an eppendorf tube and centrifuged at 2000 x g for 15 minutes (Himac Refrigerated centrifuge machine, Hitachi, Ltd., Japan). Super-natant was collected for TEAC method (100 µL), TBARS method (200 µL) and Lowry method (50 µL). The rest of supernatant was centrifuged at 15000 x g for 30 minutes. The supernatant was used for evaluating by the SOD method (100 µL) and Lowry method (50 µL).

2.7.1 TEAC method

Supernatant of brain homogenate (100 µL) was added with 1.5 mL of mixed solution containing 500 µL of 1 mM ABTS and 500 µL of 10 units/mL of horseradish peroxidase in the presence of 500 µL of 0.5 mM H_2O_2. The solution was incubated at room temperature for 6 minutes. The absorbance of radical cation $ABTS^+$ was evaluated at 734 nm using UV-160A UV-Visible recording spectrophotometer (Shimadzu, Japan). The percentage of free radical scavenging capacity of each sample was calculated as follows:

$$\% \text{ free radical scavenging capacity} = \frac{\text{Absorbance}_{blank} - \text{Absorbance}_{sample}}{\text{Absorbance}_{blank}} \times 100$$

2.7.2 TBARS method

Two hundred-µL of supernatant was pipetted into a glass tube. Twenty percent acetic acid pH adjusted to 3.5 (1.5 mL), freshly prepared 0.8% TBA (1.5 mL) and 8% SDS (200 µL) were added and incubated in a water bath at 95°C for 60 minutes and then cooled down to room temperature for 10 minutes. Then n-butanol/pyridine in ratio 15:1 v/v (2 mL) and distilled water (1 mL) were added, respectively, and centrifuged at 2000 x g at 25°C for 15 minutes. The absorb-ance of the upper part was read by UV-160A UV-Visible recording spectrophotometer at 532 nm. The standard curve was made with the same procedure using 1,1,3,3-tetraethoxy-propane (TEP) as a positive control.

2.7.3 SOD method

One hundred-µL of the supernatant was pipetted into a glass tube. SOD reagent, 0.3 mM xanthine, 400 mM Na_2CO_3, 0.6 mM EDTA, 150 µM nitroblue tetrazolium (NBT), and 1 g/L of bovine serum albumin (BSA), was added in the volume of 490 µL. To begin the reaction, 10 µl of 167 units/L xanthine oxidase was added. The solution was incubated at room temperature for 20 minutes. The reaction was stopped by adding 200 µL of 0.8 mM copper (II) chloride ($CuCl_2$). The absorbance was read at 560 nm using UV-160A UV-Visible recording spectrophotometer. The standard curve was made with the same procedure by using porcine erythrocyte SOD enzyme as a positive control. The percentage of SOD activity of each sample was calculated as follows:

$$\% \text{ SOD activity} = \frac{\text{Absorbance}_{blank} - \text{Absorbance}_{sample}}{\text{Absorbance}_{blank}} \times 100$$

2.8 Statistical analysis

Results were expressed as mean ± S.E.M. Two-way ANOVA followed by Tukey's multiple comparisons test was used to analyze the locomotor activity data performed on the experimental days 1, 14, 17 and 20 with time and treatment as main factors. In apomorphine challenge, results from the experimental day 21 were compared to those on the experi-mental day 20 using student's paired t-test. One-way ANOVA followed by Tukey's multiple comparisons test was used to analyze effects of treatment on free radical scavenging

capacity, MDA level and SOD activity. P-value < 0.05 was considered to indicate a statistically significant difference. Graphpad Prism 6.0 was used for all statistical analysis.

3. Results

3.1 Locomotor activity

The locomotor velocity was measured on experimental days 1, 14, 17 and 20 using VideoMOT2 system. Two-way ANOVA showed effects of time ($F(3,126) = 169$, $p<0.0001$), treatment ($F(4,42) = 9.27$, $p<0.0001$) and time x treatment interaction ($F(12,126) = 5.28$, $p<0.001$) on rats' locomotor velocity. There was no significant difference between rats receiving ginseng extract G115 and rats receiving sterile water for 14 days. On experimental day 17, rats receiving rotenone alone, rotenone plus ginseng extract G115 (200 mg/kg) and rotenone plus ginseng extract G115 (800 mg/kg) decreased locomotor velocity, compared to controls ($p<0.0001$, $p<0.0001$ and $p<0.01$, respectively). Interestingly, pretreatment of ginseng extract G115 (400 mg/kg) can protect against rotenone-induced locomotion speed as there was a significant difference between locomotor velocity of this group of rats compared to rats receiving rotenone alone ($p<0.01$). However, on experimental day 20 (6 days after rotenone treatment), rats in PD, PD-G200, PD-G400 and PD-G800 had significantly lower locomotor velocity compared to controls ($p<0.0001$, $p<0.0001$, $p<0.0001$ and $p<0.01$, respectively) (Figure 2).

3.2 Apomorphine challenge

To examine whether rotenone treatment specifically causes presynaptic dopaminergic neuronal damage, apomorphine, a dopamine receptor agonist, was given to all rats on the experimental day 21. After receiving apomorphine, the locomotor velocity of rats in PD, PD-G200, PD-G400 and PD-G800 groups in the experimental day 21 were significantly higher than those in the experimental day 20 ($p < 0.0001$, $p < 0.001$, $p < 0.0001$ and $p < 0.001$ respectively, Student's paired t-test) (Figure 3), suggesting that rotenone treatment caused presynaptic dopaminergic neuron damage.

3.3 Free radical scavenging capacity

To determine the free radical scavenging capacity in the brain of rats receiving ginseng extract, TEAC method was used. One-way ANOVA revealed a significant difference between each group of rats ($F(4,42)=4.624$, $p<0.01$). Rotenone treatment decreased the percentages of free radical scavenging capacity in rat brain compared to controls ($p<0.05$). The brain of rats receiving ginseng extract (400 and 800 mg/kg) had significantly higher percentages of free radical scavenging capacity than the brain of rats receiving rotenone alone ($p<0.01$ and $p<0.05$, respectively) (Figure 4).

3.4 Superoxide dismutase (SOD) activity

Superoxide dismutase activity in the rat brain was determined. One-way ANOVA revealed a significant difference between each group of rats ($F(4,42)=10.57$, $p<0.0001$). Rats receiving rotenone alone (PD group) had significantly lower SOD activity compared to controls ($p<0.01$). Pretreatment with ginseng extract (400 and 800 mg/kg) increased SOD activity in the brain compared to rats receiving rotenone alone ($p<0.0001$). In addition, SOD activity of rats in PD-G800 group was significantly higher than that of rats in PD-G200 group ($p < 0.05$) (Figure 5).

3.5 Malondialdehyde (MDA) level

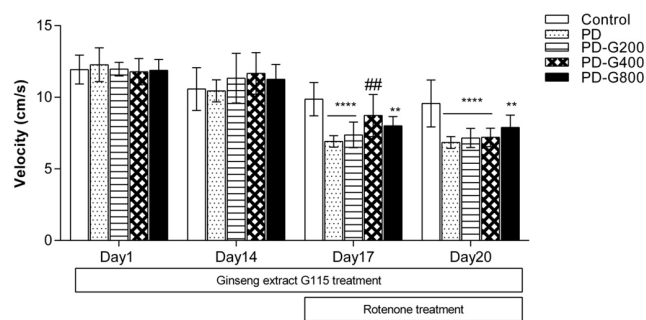To determine the lipid peroxidation in the brain homogenate, MDA levels were measured using the TBARS

Figure 2. The average locomotor velocity (cm/s) of rats receiving either sterile water (1 mL/kg p.o.) plus 2% DMSO in sunflower oil (control, n=12), or sterile water (1 mL/kg p.o.) plus rotenone (3 mg/kg i.p.) (PD, n=8), or ginseng extract G115 (200, 400 or 800 mg/kg p.o.) plus rotenone (3 mg/kg i.p.) (PD-G200, PD-G400 and PD-G800, respectively, n=9/group) in the open field test. Data are presented as mean ± S.E.M. **$p<0.01$, ****$p<0.0001$ compared to control, ## $p<0.01$ compared to PD in the same experimental day (Tukey's *post-hoc* test).
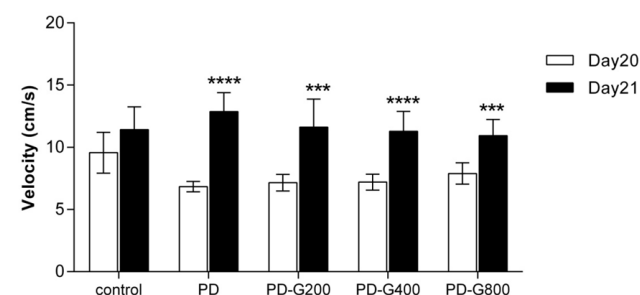

Figure 3. The average locomotor velocity (cm/s) of rats received apomorphine (1 mg/kg s.c.) in control, PD, PD-G200, PD-G400 and PD-G800 groups. *** $p<0.001$, ****$p<0.0001$ compared to the same treatment group on experimental day 20 (Student's paired t-test).
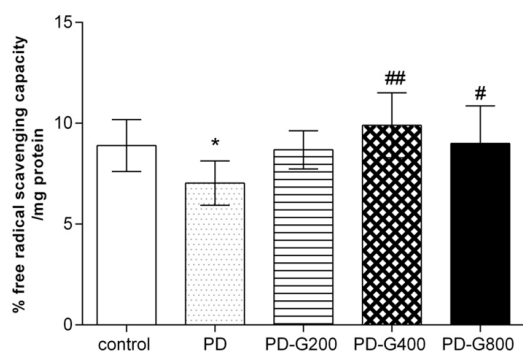
Figure 4.   Free radical scavenging capacity in the brain of rats received either sterile water (1 mL/kg p.o.) plus 2% DMSO in sunflower oil (control, n=12), or sterile water (1 mL/kg p.o.) plus rotenone (3 mg/kg i.p.) (PD, n=8), or ginseng extract G115 (200, 400 or 800 mg/kg p.o.) plus rotenone (3 mg/kg i.p.) (PD-G200, PD-G400 and PD-G800, respectively, n=9/group) using TEAC method. Data are presented as % free radical scavenging capacity/mg total protein. *$p<0.05$ compared to control, #$p<0.05$, ##$p<0.001$ compared to PD group (Tukey's *post-hoc* test).
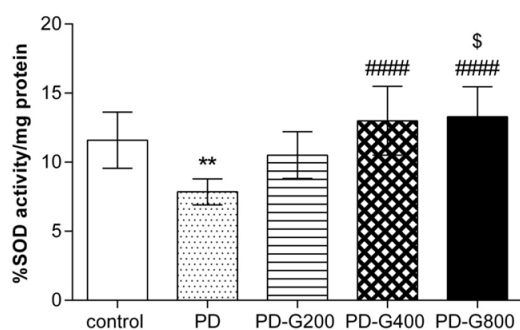

Figure 5.   Superoxide dismutase (SOD) activity in the brain of rats received either sterile water (1 mL/kg p.o.) plus 2% DMSO in sunflower oil (control, n=12), or sterile water (1 mL/kg p.o.) plus rotenone (3 mg/kg i.p.) (PD, n=8), or ginseng extract G115 (200, 400 or 800 mg/kg p.o.) plus rotenone (3 mg/kg i.p.) (PD-G200, PD-G400 and PD-G800, respectively, n=9/group). Data are presented as %SOD activity/mg protein. **$p<0.01$ compared to controls, ####$p<0.0001$ compared to PD group, $$p<0.05$ compared to PD-G200 group (Tukey's *post-hoc* test).

method. One-way ANOVA revealed a significant difference between each group of rats (F(4,42)=21.85, $p<0.0001$). Rotenone treatment increased MDA levels in the brain of rats in PD, PD-G200, PD-G400 and PD-G800 groups, compared to controls ($p<0.0001$, $p<0.0001$, $p<0.01$ and $p<0.01$, respectively). However, brain MDA levels of rats in PD-G400 and PD-G800 groups was significantly lower than those of rats in PD group ($p<0.001$). Additionally, MDA levels of rats in PD-G800 group were significantly lower than those of rats in PD-G200 ($p<0.05$) (Figure 6).

4. Discussion

Dopaminergic neurons in the substantia nigra were shown to be highly susceptible to ROS mediated damages (Testa et al., 2005). The progressive degeneration of such neurons is believed to be an important starter and enhancer of PD. Herein, we have reported for the first time that ginseng extract G115 was able to attenuate the damage in rotenone-induced parkinsonism rats. Besides, we have provided the additional information that the protective effect of ginseng extract G115 is through anti-oxidant properties.

The mechanism of rotenone-induced parkinsonism in rats is related to the conception of PD pathogenesis (Cannon et al., 2009). The mechanism of rotenone in the PD induction is a selective mitochondrial complex I inhibitor that in turn induces excessive intracellular ROS contributing to cell apoptosis (Li *et al.*, 2003; Sherer *et al.*, 2003). Moreover, exposure to rotenone and other widely used pesticides, such as organophosphates, organochlorines, and paraquat, may account as an important risk factor of Parkinson's disease in certain countries, especially Thailand (Bhidayasiri *et al.*, 2011; Pezzoli and Cereda, 2013).

In the present study, locomotor activity was initially performed on experimental day 1 before treatment given to determine the baseline movement of rats and then tested on experimental day 14 to determine the chronic effect of ginseng extract G115 on locomotor activity. The result showed that long-term treatment of ginseng extract G115 did not cause locomotor stimulation. This result is consistent with previous studies reporting that long-term treatment of Korean red ginseng extract did not affect locomotor activity in mice (Park *et al.*, 2005) and motor coordination in rats (Kupta *et al.*, 2001). Rotenone-impaired motor function in rats receiving ginseng extract G115 (400 mg/kg) before and concurrently
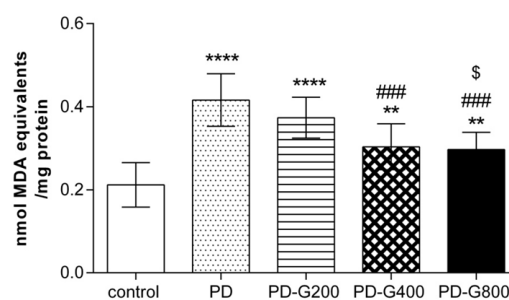

Figure 6.   MDA in the brain of rats received either sterile water (1 mL/kg p.o.) plus 2% DMSO in sunflower oil (control, n=12), or sterile water (1 mL/kg p.o.) plus rotenone (3 mg/kg i.p.) (PD, n=8), or ginseng extract G115 (200, 400 or 800 mg/kg p.o.) plus rotenone (3 mg/kg i.p.) (PD-G200, PD-G400 and PD-G800, respectively, n=9/group) using TBARS method. Data are presented as nmol MDA equivalents/mg total protein. **$p<0.01$, ****$p<0.0001$ compared to controls, ###$p<0.001$ compared to PD group, $$p<0.05$ compared to PD-G200 group (Tukey's *post-hoc* test).

with rotenone treatment had significantly improved motor function on day 17 (after 3 days of rotenone), but not day 20 (Figure 2). This suggests that pretreatment with ginseng extract G115 could delay disease progression. Similarly, Van Kampen et al (2003) previously reported that ginseng extract G115 adding in drinking water can improve motor performances in MPTP-induced parkinsonism rats. However, it was shown earlier that ginsenosides can inhibit dopamine reuptake transporter (Tsang *et al.*, 1985). Therefore ginsenosides might protect against MPTP-induced neurotoxicity by blocking MPP^+ uptake into dopaminergic neurons. As rotenone can readily penetrate through dopaminergic neurons due to its high lipophilicity, the protective effect of ginseng extract G115 found in this study is more likely to be due to its direct neuroprotective action.

Subcutaneous apomorphine challenge was used in this study as an indirect measurement of specific dopaminergic axon terminal loss. In addition, it was demonstrated that apomorphine challenge is a reliable indicator of specific neuronal damage in the nigrostriatal pathway (Duty and Jenner, 2011; Sindhu *et al.*, 2005). In this study, locomotor velocity of PD, PD-G200, PD-G400 and PD-G800 groups was improved after receiving apomorphine (1 mg/kg, s.c.), suggesting that rotenone specifically destroyed presynaptic dopaminergic neurons in the nigrostriatal pathway.

In accordance with PD pathogenesis, the neuroprotective mechanisms of ginseng extract G115 in the present study are through free radical scavenging capacity, lipid peroxidation prevention, and the antioxidant enzyme induction in the brain homogenate. The results revealed that treatment with rotenone significantly reduced free radical scavenging capacity and SOD activity, while significantly increasing the degree of lipid peroxidation in comparison to non-treated controls (Figures 4-6). Pretreatment with ginseng extract G115 (400 and 800 mg/kg) had a significantly higher free radical scavenging capacity as well as SOD activity compared to rats receiving rotenone alone. This finding indicates that ginseng extract G115 (400 and 800 mg/kg) enhanced antioxidant activities in the brain. It is noticeable that although both doses of ginseng extract G115 (400 and 800 mg/kg) treatment showed an antioxidant effect in rat brains, motor activity of rats in only PD-G400 was improved. Additionally, the activity was only attenuated from PD-treated rats but did not regain control levels. These findings suggest that the antioxidative effect of ginseng extract G115 is one of the neuroprotective mechanisms but cannot fully protect against rotenone-induced neurotoxicity. Other neuroprotective mechanisms of ginseng extract G115 should be further investigated.

In summary, the present study demonstrates the mechanism of rotenone-mediated locomotor impairment in PD rat model in relation to oxidative damages. The results of the study demonstrate the protective effect of ginseng extract G115 against such motor impairments and the underlying mechanisms involving preserved free radical scavenging and SOD activities, which in turn prevent lipid peroxidation. The findings also provide further insight into rotenone-mediated PD as well as the possible use of this extract for treatments.

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