Note

Anti-fatigue Effects of 20(S)-Protopanaxadiol and 20(S)-Protopanaxatriol in Mice

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Ginseng (*Panax ginseng* C.A. MEYER, Araliaceae), which contains protopanaxadiol-type and protopanaxatriol-type ginsenosides, has been used for inflammation, fatigue, stress, and tumor in Asian countries. Orally administered ginsenosides are metabolized to their aglycones 20(S)-protopanaxadiol (PPD) and 20(S)-protopanaxatriol (PPT) by gut microbiota. However, their anti-fatigue effects have not been studied thoroughly. Therefore, we investigated the anti-fatigue activities of PPD and PPT in mice, using the weight-loaded swimming (WLS) and the rota-rod tests. Ginseng water extract (GW), ginseng saponin fraction (GWS) and ginseng polysaccharide fraction (GWP) at concentrations of 50 and 100 mg/kg and PPD and PPT at 5 and 10 mg/kg were orally administered to mice once daily for 5 d. GW, GWS, and PPT significantly increased the WLS time, however, GWP and PPD did not cause any significant change. PPT induced the most significant increase in WLS time. PPD (10 mg/kg) and PPT (5 and 10 mg/kg) inhibited the WLS-induced increase in corticosterone, lactate, lactate dehydrogenase (LDH), and creatinine levels as well as the reduction in glucose level. PPT increased the riding time in the rota-rod test, and also inhibited corticosterone, lactate, and creatinine levels. These findings suggest that the anti-fatigue effect of ginseng may be attributable to its saponins, particularly PPT, rather than to its polysaccharides.

Key words *Panax ginseng*; 20(S)-protopanaxadiol; 20(S)-protopanaxatriol; fatigue

Fatigue is described as the lack of energy and motivation to initiate and sustain voluntary physical and mental activities.¹⁾ Physical fatigue is the transient inability of a muscle to maintain physical performance and is made more severe by physical stress. Mental fatigue is a transient decrease in cognitive performance. Most cases of fatigue may be attributed to lifestyle factors such as alcohol abuse and excessive physical activity, medical conditions such as multiple sclerosis and Parkinson's disease, or psychological problems such as anxiety and stress. Therefore, natural products including ginseng have been studied with the aim of developing anti-fatigue drugs to improve athletic ability, delay fatigue, and enhance the elimination of fatigue in humans.^{2–6)}

Ginseng (the root of *Panax ginseng* C.A. MEYER, Araliaceae) has been widely used as a traditional Chinese medicine for enhancing body strength, recovering physical balance, and stimulating metabolic function in Asian countries. It contains various active constituents such as ginsenosides, polysaccharides, polyacetylenes, phenolic compounds, and peptides.⁷⁾ Of constituents, ginsenosides have been reported to show biological activities, including anti-inflammatory, anti-fatigue, anti-stress, and anti-tumor activities.^{8–12)} The ginsenosides are classified as protopanaxadiols or protopanaxatriols. Orally administered protopanaxadiol-type and protopanaxatriol-type ginsenosides are metabolized to 20(S)-protopanaxadiol (PPD) via compound K and 20(S)-protopanaxatriol (PPT) via ginsenoside Rh1, respectively, by gut microbiota.^{13–16)} Therefore, an understanding of the pharmacological effects of ginseng requires elucidation of the pharmacological activities of PPD and PPT. However, the anti-fatigue effects of the ginsenoside metabolites have not been studied thoroughly.

In a preliminary study, we found that the ginseng saponin fraction (GWS) exhibited a more potent anti-fatigue activity than the ginseng polysaccharide fraction (GWP), which has anti-fatigue activity.⁴⁾ Therefore, we investigated the anti-fatigue activities of the main metabolites PPD and PPT of ginsenosides produced by gut microbiota in mice.

MATERIALS AND METHODS

Materials 20(S)-Protopanaxadiol (PPD, purity $\geq 98\%$ by HPLC) and 20(S)-protopanaxatriol (PPT, purity $\geq 98\%$ by HPLC) were purchased from Ambo Institute (Daejeon, Korea) (Fig. 1). Assay kit for corticosterone was purchased from Abcam (Cambridge, MA, U.S.A.). Assay kits for serum lactate, lactate dehydrogenase (LDH), free fatty acid (FFA) and creatinine were purchased from Bioassay system (CA, U.S.A.). And blood glucose was assayed using Accu-chek inform II system (Mannheim, Germany).

Plant Material and Extract The roots of *Panax ginseng* C. A. MEYER were collected from Geumsan province in Korea, in September 2014, and identified by Nam-Jae Kim, a coauthor of the present study. A voucher specimen (KHUP20140920-1) was deposited at the College of Pharmacy, Kyung Hee University.

The air-dried roots of *P. ginseng* (200 g) were extracted with distilled water at 100°C for 2 h and the extract was concentrated and freeze-dried to obtain the ginseng water extract (GW). The GW (20 g) was then suspended in distilled water and fractionated three times with butanol and this soluble fraction was concentrated, freeze-dried, and designated as the ginseng saponin (GWS).¹⁷⁾ The water soluble fraction was

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(A)

(B)

Fig. 1. Chemical Structures of PPD (A) and PPT (B)

precipitated by adding 70% ethanol. After centrifugation, the precipitate was suspended in distilled water, and the supernatant was concentrated, freeze-dried, and designated as the water-soluble polysaccharide (GWP).⁴⁾

Animals Male ICR mice (7 weeks-old, 26–28 g) were purchased from Samtako Biokorea (Seoul, Korea) and acclimated for 1 week before use. All animals were maintained under a constant temperature ($24 \pm 2^\circ\text{C}$) and humidity ($60 \pm 10\%$) with an alternating 12 h light–dark cycle. They were fed on standard laboratory chow (Samyang Co., Seoul, Korea) with tap water *ad libitum*. All experiments were performed in accordance with the National Institutes of Health and Kyung Hee University guides for Laboratory Animals Care and Usage. The protocol was approved by the Institutional Animal Care and Use Committee of the Kyung Hee Medical Center and Kyung Hee University.

Each group consisted of 7 mice for all experiments. The mice were randomly divided 7 groups: Group 1 (control) was given into distilled water; Groups 2 and 3 were given into 50 and 100 mg/kg of GW; Groups 4 and 5 were given into 50 and 100 mg/kg of GWS; Groups 6 and 7 were given into 50 and 100 mg/kg of GWP. To study for the anti-fatigue effects of PPD and PPT, mice were also divided 6 groups: Groups 1 and 2 (normal and control) were given into 0.5% carboxymethylcellulose (CMC) solution; Groups 3 and 4 were given into 5 and 10 mg/kg of PPD; Groups 5 and 6 were given into 5 and 10 mg/kg of PPT. Each sample was orally administered once daily for 5 d. The weight-loaded swimming (WLS) and rota-rod tests were conducted once every 2 d 30 min after the sample administration except normal group.

WLS Test The WLS test was performed with slight modifications according to the previous method described by Tan *et al.*¹⁸⁾ Briefly, after each mouse was weighed, a lead block (10% of the body weight) was loaded onto its tail. The mice were then dropped individually into an acrylic plastic pool ($40 \times 25 \times 25$ cm) filled with fresh water maintained at

$18 \pm 1^\circ\text{C}$, and approximately 20 cm deep. The swimming time of mice was calculated from the time began to swim up to the time exhibited exhaustion, which was determined as a loss of coordinated movements and failure to return to the surface within 3 s. The length of the swimming time to exhaustion was evaluated as the degree of fatigue.

Rota-Rod Test The rota-rod test was performed with slight modifications as previously described.¹⁹⁾ Before the test, all mice were trained on running at a speed of 30 rpm for 60 s during 2 d to adapt to the rota-rod instrument (KN-75, Natume, Japan). Each mouse was placed inside a rota-rod spinning at a speed of 70 rpm. The mice were allowed to run in the rota-rod until they were exhausted to the point of dropping from the rod, and the total running time was recorded.

Serum Analysis The mice were sacrificed after the final WLS and rota-rod tests, and blood samples were collected from the carotid artery and centrifuged at 3000 rpm for 5 min at 4°C . The serum was separated, and biochemical parameters including corticosterone, lactate, LDH, FFA, and creatinine levels were measured using a commercial kit according to the manufacturer's protocol. Lactate was determined by LDH catalyzed oxidation of lactate, in which the formed nicotinamide adenine dinucleotide (NADH) reduces a thiazolyl blue tetrazolium bromide (MTT) reagent, and measured at 565 nm. LDH was determined by reduction of the tetrazolium salt MTT in a NADH-coupled enzymatic reaction and measured at 565 nm. FFA is enzymatically converted to acyl-CoA and subsequently to H_2O_2 . The resulting H_2O_2 reacts with a dye and the product was measured at 570 nm. Creatinine was determined by improved Jaffe method and it utilized picrate that forms a red colored complex with creatinine and was measured at 510 nm.

Statistical Analysis All data are expressed as the mean $\pm$ standard error of the mean (S.E.M.) The significant difference was analyzed by one-way ANOVA followed by a Dunnett's test and two-way ANOVA followed by Bonferroni post-test for multiple comparisons. Statistical significance was set at $p < 0.05$.

RESULTS

Effects of GW, GWS and GWP on WLS Test To understand the anti-fatigue activities of the constituents of ginseng, we extracted ginseng with distilled water and obtained GW, which was further fractionated to obtain GWS and GWP. We then measured the anti-fatigue effects of them in mice using the WLS test. The GW (100 mg/kg) and GWS (50 and 100 mg/kg) significantly increased the WLS time. Treatment with GWS (50 and 100 mg/kg) increased the WLS time by 40.0 and 47.8% on day 5 compared with that of the control group (Fig. 2A, $p < 0.01$). However, GWP did not cause any significant change.

Effects of PPD and PPT on WLS Test The ginsenosides are metabolized to their respective aglycones PPD and PPT by gut microbiota. Based on a preliminary experiment and previous studies,²⁰⁾ we investigated the anti-fatigue effects of the aglycones PPD and PPT at the dosages of 5 and 10 mg/kg in mice using WLS test. Treatment with PPT (5 and 10 mg/kg) significantly increased the WLS time of the mice. Particularly, treatment with PPT (10 mg/kg) increased the WLS time by 57.3% on day 5 compared with that of the control group (Fig. 2B, $p < 0.01$). However, the PPD did not cause any significant

Fig. 2. Effects of Ginseng Extracts, PPD and PPT on the WLS Time in Mice

(A) Mice were orally administered with ginseng water extract (GW), ginseng saponin fraction (GWS) and ginseng polysaccharide fraction (GWP) (50 and 100 mg/kg) once daily for 5 d. (B) Mice were orally administered with PPD and PPT (5 and 10 mg/kg) once daily for 5 d. The WLS test was conducted once every 2 d 30 min after the sample administration. The data were expressed as the mean $\pm$ S.E.M. of 7 mice; * p < 0.05, ** p < 0.01, *** p < 0.001 vs. control group.

Table 1. Effects of PPD and PPT on Serum Biochemical Parameters after the WLS Test in Mice

	Dose (mg/kg, <i>per os</i> (p.o.))	Corticosterone (ng/mL)	Glucose (mg/dL)	Lactate (mm)	LDH (U/L)	FFA (μ M)	Creatinine (mg/dL)
Normal		42.1 $\pm$ 4.3	216.8 $\pm$ 9.7	5.9 $\pm$ 0.6	2721.6 $\pm$ 258.3	549.8 $\pm$ 28.3	0.13 $\pm$ 0.01
Control		151.8 $\pm$ 6.1 ^{###}	176.6 $\pm$ 15.1 ^{##}	12.1 $\pm$ 1.2 ^{##}	3705.0 $\pm$ 339.9 ^{##}	656.3 $\pm$ 44.4 ^{##}	0.20 $\pm$ 0.02 ^{##}
PPD	5	144.9 $\pm$ 5.9	190.7 $\pm$ 12.6	11.0 $\pm$ 0.5	3406.6 $\pm$ 251.3	657.8 $\pm$ 35.8	0.19 $\pm$ 0.02
PPD	10	127.8 $\pm$ 12.5*	202.0 $\pm$ 12.2*	10.3 $\pm$ 1.0*	3180.2 $\pm$ 146.7*	554.3 $\pm$ 50.6*	0.16 $\pm$ 0.01*
PPT	5	114.8 $\pm$ 8.7*	202.0 $\pm$ 12.7*	10.4 $\pm$ 1.1*	3283.9 $\pm$ 181.5*	573.8 $\pm$ 40.8*	0.16 $\pm$ 0.01*
PPT	10	80.6 $\pm$ 8.7**	213.8 $\pm$ 10.7**	7.7 $\pm$ 0.5**	2990.8 $\pm$ 133.3**	534.3 $\pm$ 47.0**	0.15 $\pm$ 0.01**

Mice were orally administered with PPD and PPT (5 or 10 mg/kg) once daily for 5 d. After the final WLS test, the mice were sacrificed and the blood samples were collected from carotid artery. The data were expressed as the mean $\pm$ S.E.M. of 7 mice; ^{##} p < 0.01, ^{###} p < 0.001, vs. normal group; * p < 0.05, ** p < 0.01 vs. control group.

increase.

Performance of WLS by the mice decreased the glucose level and increased corticosterone, lactate, LDH, FFA, and creatinine levels compared with the normal mice (Table 1, p < 0.01). Treatment of PPD and PPT attenuated the induction of serum levels of the biochemical parameters. In particular, treatment with PPT (10 mg/kg) led the serum levels to normal (p < 0.01). The attenuating effect of the PPT against enhanced

fatigue-related biochemical parameters was more potent than that of the PPD.

Effect of PPT on Rota-Rod Test To confirm anti-fatigue activity of PPT, we also measured its effect on the riding time using rota-rod test. PPT (10 mg/kg) significantly increased the riding time for 5 d (Fig. 3). Treatments with PPT at 5 and 10 mg/kg of the concentrations increased the riding time by 28.7 and 47.8% on day 5 compared with that of the control

Table 2. Effect of PPT on Serum Biochemical Parameters after the Rota-Rod Test in Mice

	Dose (mg/kg, <i>p.o.</i>)	Corticosterone (ng/mL)	Lactate (mM)	FFA (μ M)	Creatinine (mg/dL)
Normal		65.2 $\pm$ 5.2	6.3 $\pm$ 0.5	539.3 $\pm$ 31.2	0.18 $\pm$ 0.01
Control		166.4 $\pm$ 13.9 ^{###}	10.6 $\pm$ 0.8 [#]	627.6 $\pm$ 47.6 [#]	0.26 $\pm$ 0.02 ^{##}
PPT	5	146.1 $\pm$ 9.6*	7.7 $\pm$ 0.6*	571.3 $\pm$ 26.3	0.22 $\pm$ 0.02
PPT	10	129.0 $\pm$ 11.6**	7.1 $\pm$ 0.5*	538.3 $\pm$ 49.7*	0.21 $\pm$ 0.02*

Mice were orally administered with PPT (5 and 10 mg/kg) once daily for 5 d. After the final rota-rod test, the mice were sacrificed and the blood samples were collected from carotid artery. The data were expressed as the mean $\pm$ S.E.M. of 7 mice; [#] p <0.05, ^{##} p <0.01, ^{###} p <0.001, vs. normal group; * p <0.05, ** p <0.01 vs. control group.

Fig. 3. Effect of PPT on the Rota-Rod Riding Time in Mice

Mice were orally administered with PPT (5 and 10 mg/kg) once daily for 5 d. The rota-rod test was conducted once every 2 d at 30 min after the sample administration. The data were expressed as the mean $\pm$ S.E.M. of 7 mice; * p <0.05, ** p <0.01 vs. control group.

group (p <0.01). Performance of the rota-rod riding by the mice increased serum corticosterone, lactate, FFA, and creatinine levels compared with the normal mice (Table 2). Treatment of PPT attenuated the induction of these serum levels.

DISCUSSION

Fatigue causes various disorders associated with bio-regulation of the autonomic nervous, endocrine, and immune systems and can be divided into two categories: physical fatigue caused by such things as forced exercise or swimming; mental fatigue caused by sleep deprivation, *etc.*^{21,22} Particularly, physical fatigue is caused by the depletion of energy sources, including a decrease in glycemic levels. The excessive accumulation of metabolites such as blood lactate, FFA, creatinine and ammonia leads to fatigue.²³ Blood lactate is produced during anaerobic glycolysis to obtain sufficient energy during high-intensity exercise. Lactate reduces the pH of muscle and blood and induces various biochemical processes such as oxidative stress. LDH and creatinine are important indicators of muscle damage. The increase in anaerobic glycolysis leads to a compensatory increase in fat mobilization.⁶ The WLS test is commonly used as animal model for evaluating the extent of physical fatigue and a strenuous exercise leading to physical stress triggering the activation of pituitary adrenocortical activity.^{24–26}

Saponins and polysaccharides isolated from natural products have anti-fatigue activity: saponins from *Astragalus membranaceus* and *Panax ginseng* and polysaccharides from *Morinda officinalis* and *Radix Rehmanniae* Preparata exhibit the anti-fatigue activities.^{18,27–29}

Ginseng has been used for a long time in Asian countries to treat many disorders, including aging, fatigue, stress, tumor,

and insomnia.^{16,30} Its bioactive constituents are saponins, polysaccharides, and proteins.^{16,17} Ginseng and its constituents show anti-fatigue effect.^{3,4,6,31,32} When ginseng is orally administered, its hydrophilic constituents are metabolized to hydrophobic compounds by gut microbiota.^{13–16} For example, protopanaxadiol-type and protopanaxatriol-type ginsenosides are metabolized to their aglycones PPD and PPT, respectively. Nevertheless, the anti-fatigue effects of PPD and PPT have been not studied.

In the present study, we investigated the anti-fatigue activities of GW, GWS, and GWP. GW and GWS potently increased the WLS time of mice as shown in previous studies. However, we found that the mice treated with GWP did not show an extended WLS time compared to the untreated mice. Furthermore, the final metabolite of the ginseng saponins, PPT caused a significant increase in the WLS time of the mice, but not PPD. Nevertheless, PPT and PPD prevented the induction of blood corticosterone, lactate, LDH, FFA, and creatinine levels as well as the reduction of glucose level by the WLS. However, the protective effect of the PPT was more potent than that of PPD. PPT also increased the riding time in rota-rod and ameliorated the levels of blood biological parameters as corticosterone, lactate, and creatinine.

Based on these findings, we propose that ginseng may exhibit anti-fatigue effect, which is attributable to the activity of the PPT metabolite of ginsenosides.

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Conflict of Interest The authors declare no conflict of interest.

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