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Original Article

Rh2-enriched Korean Ginseng Ameliorates Chronic Fatigue in a Forced Exercise mouse model

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Objectives: We evaluated the anti-fatigue effects of Rh2-enriched Korean ginseng (Ginseng Rh2+) using a forced exercise-induced chronic fatigue mouse model.
Methods: ICR male mice were subjected to running wheel for 1 h, 5 days/week during 4 weeks, and running velocity was gradually increased. Each running session was followed by oral administration of distilled water, Ginseng Rh2+ (150 or 300 mg/kg), or N-acetyl-L-cysteine (NAC, 100 mg/kg) 1 h later. The exercise tolerance and forced swimming test were performed to evaluate the fatigue condition.
Results: Chronic forced exercise reduced the physical activity, as evidenced by the behavioral tests, which were notably ameliorated by Ginseng Rh2+ treatment. Ginseng Rh2+ treatment also attenuated the alterations of energy metabolism and oxidative stress in skeletal muscle tissues and/or sera, including malondialdehyde (MDA), lactate concentration and its related factors (lactate dehydrogenase, blood urea nitrogen, and glucose levels).
Conclusion: These findings strongly suggest that Ginseng Rh2+ exerts a potent anti-fatigue effect through modulation of energy metabolism and oxidative response.

Key Words : Chronic fatigue; Oxidative stress; Lactate; Ginseng Rh2+

Introduction

Panax ginseng is a most representative medicinal herb which is widely used worldwide as an adaptogens. P. ginseng generally has a good safety profile, and it's some adverse effects are mild and transient[1)]. The pharmacological active compounds of P. ginseng are ginsenosides, and approximately 40 ginsenosides have been identified. To enforce the biological effects and/or minimize the adverse effects, fresh ginseng is processed into red ginseng, and various processing methods for specific ginsenoside-enriched products have been devised[2)]. Numerous studies have demonstrated the pharmaceutical effects of red ginseng on psycho- and/or physical -stress[3)], immune deficiency[4)], metabolic dysfunction[5)], and cancer[6)].

On the other hand, chronic fatigue is a debilitating condition by persistent tiredness over 6 months, which has about 10% of prevalence in general

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population worldwide[7]. Although pathogenesis of chronic fatigue is not fully elucidated, accumulated evidences suggest an association with the immune and endocrine system, and oxidant-antioxidant balance[8]. In addition, disruption of energy homeostasis is commonly observed in the chronic fatigue and fatigue-related patients[9]. Previous studies reported that repetitive muscle contraction leads the depletion of muscle ATP and dysregulation of glycolysis/gluconeogenesis, which is associated with muscle fatigue[10,11]. Therefore, disability of energy metabolism in skeletal muscle has been considered as potential treatment targets for fatigue disorders[12].

Ginsenoside Rh2 belongs to protopanaxadiols which is found only in red ginseng. Previous reports reported the pharmaceutical activities of ginsenoside Rh2 such as prevention of metabolic disorders[13], inhibition of inflammation-mediated neuronal degeneration[14], and anti-cancer property[15,16]. Accordingly, ginsenoside Rh2 would be a target of red ginseng-processing technology.

In order to get the initial evidence for the potential of Rh2-enriched red ginseng (called as Ginseng Rh2+), we have tried to examine if Rh2-enriched red ginseng has an anti-fatigue effect. In this study, we evaluated the anti-fatigue effect Ginseng Rh2+ using a mouse model of forced exercise-induced chronic muscle fatigue, and investigated its corresponding mechanisms regarding modulation of energy metabolism and oxidative stress.

Materials and methods

1. Preparation of Ginseng Rh2+

The fresh ginseng root (Panax ginseng C.A. Meyer, 5-6 years old, cultivated at *Pocheon-gun*, South Korea) was steamed for 2 hrs at 100°C, which was followed by drying 48 hrs at 100°C. 100kg of this red ginseng (crushed) were boiled for 24 hrs at 100°C. After incubating with enzymes (0.9% lactase and 0.1% β-glucosidase) for 48 hrs at 40°C, 100kg of dried red ginseng (Ginseng Rh2+) were attained, and the filtered using 20 meshes. The quantity of Rh2 including other ginsenosides (Rb1, Rg3, Rh1, and compound K) between red ginseng and Ginseng Rh2+ was analyzed using Acquity UPLC system (Waters, Milford, MA) with an Acquity BEH C_{18} high-performance liquid chromatography (HPLC) column (Fig. 1A). Above samples were manufactured and provided by MICO (Co.).

2. Chemicals and reagents

The following reagents were purchased from Sigma (St. Louis, MO, USA): N-acetyl L cysteine (NAC), Bicinchoninic acid solution, copper (II) sulfate solution, and thiobarbituric acid (TBA). Hydrogen peroxide was purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan). Methanol and *N*-butanol was purchased from J.T.Baker (Mexico City, Mexico),

3. Animals and chronic forced-exercise protocol

Forty-eight, specific-pathogen-free, male ICR mice (34-36g, 10 week old, Dae-Han Biolink, Chungcheongbuk-do, Korea) were used. The mice had *ad libitum* access to water and food pellets (Cargill Agri Furina, Gyeonggido, Korea), and were housed in a room maintained at 23 ± 2°C with a 12-h: 12-h light-dark cycle. All animal experimental protocols were approved by the Institutional Animal Care and Use Committee of Daejeon University, and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH).

After acclimatization for 1 week, the mice were randomly divided into five group (n = 8/group): naïve, control, Ginseng Rh2+ (150, 300 mg/kg), and

positive control (100 mg/kg, NAC). Ginseng Rh2+ and NAC were dissolved in distilled water. All mice were orally administered distilled water (naïve and control), Ginseng Rh2+, or NAC using a sonde for 4 weeks, and 1 h after forced-running wheel exercise. Dosage of drugs was decided based on previous studies (Refs). Details experimental design is shown in Fig. 1B.

4. Chronic forced-exercise procedure

Except for naïve group, all mice were subjected to chronic forced exercise using a motorized running wheel (20-cm diameter by 5-cm width; Shandong Yiyuan Technology Development Co., Shandong, Binzhou, China). Initially all mice were adapted to running wheel equipment for 30 min at a velocity of 6 m/min. During the first week, the exercise procedure was performed at a velocity of 10 m/min for 60 min, followed by gradual increases to 12, 14, and 16 m/min on 2, 3, and 4 weeks, respectively. Exercise procedure was performed under 1.0 or 2.0 mA electric shocks for motivate to perform the exercise. During exercise, equipment considered as exhaustion when mice were given up to 10 immediate electric shocks, and then running wheel was stopped automatically for 30 s. The number of electric shocks, running speed, and exercise times were recorded automatically. Mice were undergoing behavioral tests consisting of an exercise tolerance test on day 29, and a forced swimming test on day 30.

5. Exercise tolerance test and number of electric shock

Forty-eight mice, including the naïve group, were run on the exercise wheel at a velocity of 25 m/min until exhaustion, and the running times recorded. The number of electric shocks administered during

A

Ginsenoside compounds	Rb1	Rg3-R	Rg3-S	Rh1	Rh2-R	Rh2-S	Compound K
Red ginseng (mg/g)	3.85	0.20	0.32	0.98	0.00	0.82	0.83
Ginseng Rh2+ (mg/g)	1.50	3.71	2.35	3.20	0.45	2.95	589

B

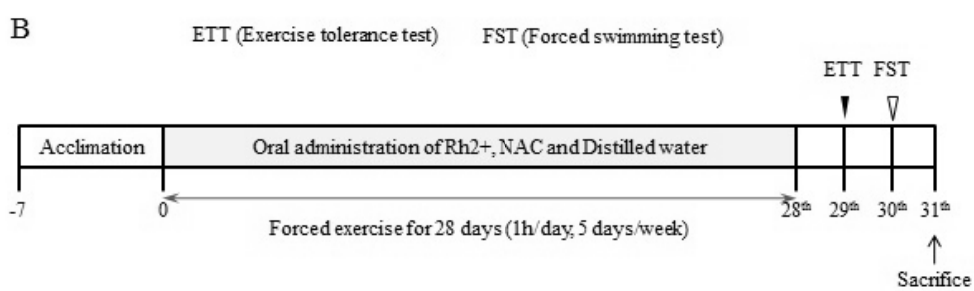

Fig. 1. Quantitative analysis of Ginseng Rh2+ and experimental scheme.
Ginsenoside compounds containing in Ginseng Rh2+ and general red ginseng were quantified (A). The diagram depicts the experimental design used in this study (B); including exercise tolerance tests (ETT) and forced swimming tests (FST).

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exercise over the 3 days prior to the exercise tolerance test was used to calculate the average of electric shock number.

6. Forced swimming test

The forced swimming test was performed using a cylindrical container 20 cm in diameter by 30 cm high. The container was filled with water (24 ± 1°C) to a depth of 20 cm. Mice were individually placed into each container and allowed to swim for 5 min. Latency time was recorded when a mouse first showed an immobile position, defined as a mouse floating in an upright position for > 3 s, making only small movements to keep its head above water. The number of immobile positions was recorded over the course of 5 min.

7. Sample preparation

Mice were sacrificed under ether anesthesia following behavioral tests on the day 31. Serum was separated from clotted blood by centrifugation at 3000 × g for 15 min at 4°C, and the skeletal muscle was isolated immediately. Sera and skeletal muscle tissues were stored at -80°C until further use.

8. Determination of MDA levels in skeletal muscle

The lipid peroxidation level in skeletal muscle was determined using a thiobarbituric acid reactive substance (TBARS) method described previously[17], which was based upon accumulation of malondialdehyde (MDA) products. Briefly, a 10% (w/v) skeletal muscle tissue homogenate was prepared in ice-cold 1.15% KCl, after which 130 μL of the homogenate were mixed with 80 μL of 1% phosphoric acid and 260 μL of 0.67% thiobarbituric acid (TBA). The mixture was then incubated at 100°C for 45 min, followed by the addition of 1.03 mL of n-butanol. The mixture was then vortexed and centrifuged at 3,000 × g for 15 min at 4°C, and absorbance at 535 and 520 nm measured using a UV spectrophotometer. TBARS concentrations were calculated using 1,1,3,3 -tetraethoxypropane (TEP) as the standard, and expressed as μmol/mg protein.

9. Determination of l-lactate levels in serum and skeletal muscle tissue, and LDH, glucose, and BUN levels in serum

L-lactate concentrations in serum and skeletal muscle were determined using a commercially available l-lactate assay kit (Eton Bioscience Inc., San Diego, CA, USA). Briefly, a 10% (w/v) skeletal muscle tissue homogenate was prepared in 80% ethanol, and then sera and homogenates were used in according to the manufacturer's protocol. The absorbance at 490 nm was measured using a UV spectrophotometer. Lactate dehydrogenase (LDH), blood urea nitrogen (BUN), and glucose levels were determined using an autoanalyzer (Chiron, Emeryville, CA, USA).

10. Statistical analyses

All data are expressed as means ± standard deviation (SD). Differences between groups were evaluated by one-way analysis of variance (ANOVA) followed by a post hoc correction of multiple comparisons using Fisher's LSD t-test. All analyses were performed using IBM SPSS statistics software, ver. 20.0 (SPSS Inc., Chicago, IL, USA). In all analyses, $P < 0.05$ was taken to indicate statistical significance.

Results

1. Effects of Ginseng Rh2+ on the exercise tolerance test

Exercise tolerance was significantly declined in the control group compared with the naïve group ($P < 0.05$), whereas these exercise disability was

significantly ameliorated by Ginseng Rh2+ treatment compared with the control group ($P < 0.01$ for 300 mg/kg; Fig. 2A). Consistent with above result, Ginseng Rh2+ treatment was associated with significantly lower average electronic shock numbers compared with the control group in the 3 days prior to the exercise tolerance test ($P < 0.01$ for 150 mg/kg, $P < 0.05$ for 300 mg/kg; Fig. 2B).

2. Effects of Ginseng Rh2+ on the forced swimming test

Chronic forced exercise significantly shortened latency time, and prolonged immobility time compared with the naïve group ($P < 0.001$ in both). Ginseng Rh2+ treatment significantly ameliorated not only the shortened latency time ($P < 0.01$ for 150 mg/kg, $P < 0.05$ for 300 mg/kg; Fig. 2C) but also the prolonged immobility time ($P < 0.01$ for both 150 and 300 mg/kg; Fig. 2D). NAC have shown also significant effects on the forced swimming test.

3. Effects of Ginseng Rh2+ on MDA levels in skeletal muscle

Chronic forced exercise significantly elevated the MDA adduct in skeletal muscle compared with the naïve group (by approximately 3-fold, $P < 0.001$). This elevation was significantly attenuated in Ginseng Rh2+ treatment groups compared with the control group ($P < 0.01$ for both 150 and 300 mg/kg; Fig. 3). NAC exerted similar positive effects on the skeletal muscle MDA level.

4. Effects of Ginseng Rh2+ on l-lactate levels in serum and skeletal muscle

L-lactate concentrations in serum and skeletal muscle were significantly increased by chronic

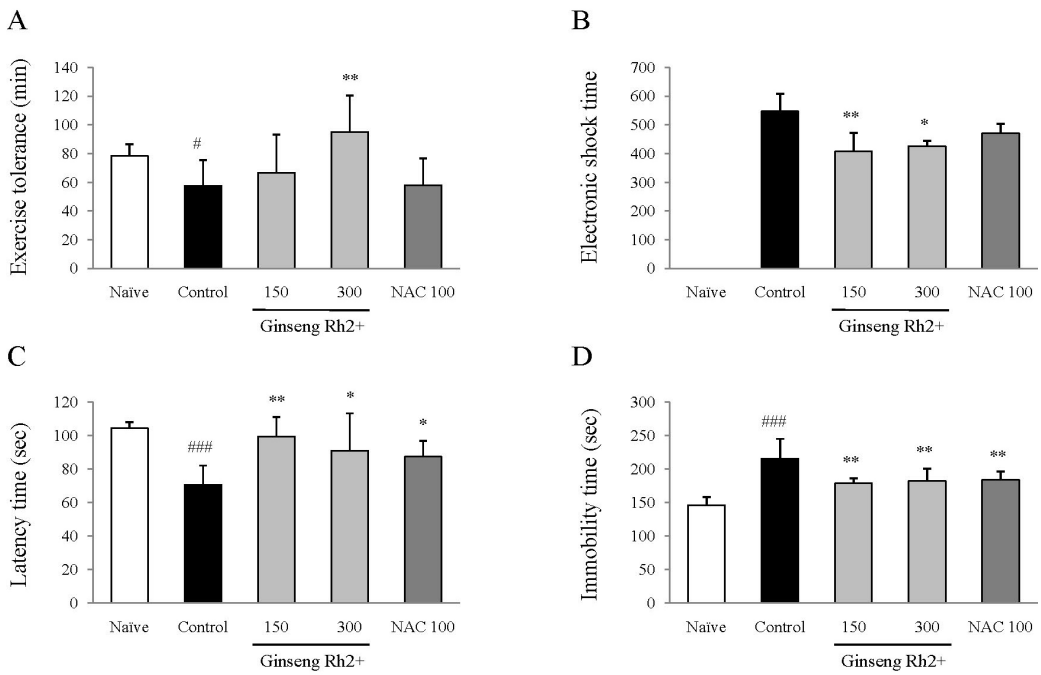

Fig. 2. Behavioral analysis of the anti-fatigue effects of Ginseng Rh2+.
On day 29, mice were subjected to exercise tolerance tests to evaluate fatigue (A). Average electronic shock times during runs on days 26-28 (B). Latency time (C) and immobility time (D) were recorded during the forced swimming test on day 30. Data are presented as means ± SD (n = 8). $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ compared with the naïve group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control group.

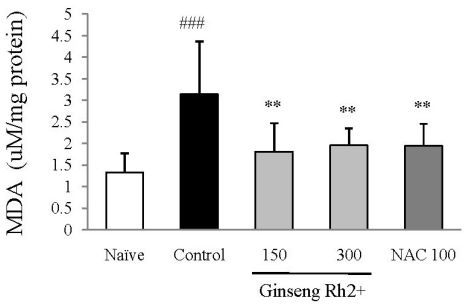

Fig. 3. Change in skeletal muscle MDA level.
Lipid peroxidation in skeletal muscle tissue was determined by response to TBA-MDA adducts. Data are presented as means ± SD (n = 8). $^{###}P < 0.001$ compared with the naïve group; $^{**}P < 0.01$ compared with the control group.

forced exercise compared with the naïve group ($P < 0.001$ and $P < 0.01$, respectively). Ginseng Rh2+ treatment significantly decreased the concentrations of l-lactate in both serum and skeletal muscle compared with the control group ($P < 0.01$ for both 150 and 300 mg/kg in serum, $P < 0.05$ for 300 mg/kg in skeletal muscle; Fig. 4A). Similar effects were observed in the NAC treatment group.

5. Effects of Ginseng Rh2+ on serum LDH, BUN, and glucose concentrations

Chronic forced exercise significantly increased serum levels of LDH, BUN and glucose compared with the naïve group ($P < 0.05$ for all biomarkers). The elevations in LDH and glucose level were significantly attenuated by Ginseng Rh2+ treatment ($P < 0.05$ for 300 mg/kg in LDH, $P < 0.05$ for both 150 and 300 mg/kg in glucose; Fig. 4B and D). The BUN level was slightly decreased in Ginseng Rh2+ treatment group, but there was no significant difference (Fig. 4C). NAC conferred effects similar to those of Ginseng Rh2+.

Discussion

World market of ginseng including fresh, white, and red ginseng is estimated at approximately 2,084 million USD at 2009[18]. Along with this huge demand for ginseng worldwide, novel technologies are still developing according to consumer's requirement such as specific ginsenoside-enriched ginseng. Ginsenoside Rh2 is contained in the red ginseng, which is well-known to having an anti-tumor growth effect against various cancer cell lines[19,20]. Ginseng Rh2+ recently presented several pharmaceutical properties; including anti-allergic inflammation in airway[21], cardio-protective effects in both animal and cell model[22], and inhibitory effects against amyloid β-derived memory impairment[23]. Ginseng Rh2+ contained approximately 3.4 mg/g of Rh2 content (Fig. 1A). This result showed a high-rich quantity of ginsenoside Rh2 comparing to general Korean ginseng[24].

To investigate the anti-fatigue effects of Ginseng Rh2+, we adopted an animal model of chronic fatigue by repetitive forced exercise for 4 weeks. Based on the previous experiments and clinical dose (2g daily), we decided its concentration. Chronic forced exercise is commonly used to explain the chronic fatigue and fatigue-associated disorders, and applied to evaluate the anti-fatigue effects of candidates in animal model[25]. Here, we used the NAC as a positive control because its potent anti-fatigue effects were already proved[26]. As expected, chronic forced exercise leaded to physical fatigue behavior, as evidenced by the both exercise tolerance test and forced swimming test. Typical symptom of chronic fatigue is a physical inactivity[27], which is often observed in patients with fatigue -related disorders[28]. In this study, the Ginseng Rh2+ treatment remarkably ameliorated the fatigue-related inactive behavior in both exercise tolerance and forced swimming tests (Fig. 2A-D).

Consistent with fatigue behavioral condition, reduction of physical activity is caused by dysfunction of energy metabolism in the peripheral muscle. During contraction of muscles, myocytes generate the anaerobic ATP to maintain constant physical

activity through glycolysis process[29]. Eventually, the repeated intense exercise disrupts glycolysis/ gluconeogenesis homeostasis, which is well-known as one of the pathogenesis in the fatigue disorders[30]. We examined the effects of ginseng Rh2+ on key modulators of the energy metabolism in the skeletal muscle. As expected, Ginseng Rh2+ treatment attenuated the elevation of lactate production in both serum and skeletal muscle tissue (Fig. 4A). As a linking factor in lactate accumulation, the LDH activity and glucose levels were elevated in serum, which may be an indicator of activate glycolysis. Then these parameters were also attenuated by Ginseng Rh2+ treatment (Fig. 4B and D). A byproduct of energy metabolism, the elevated BUN level was slightly reduced by Ginseng Rh2+ treatment, but significant difference was not observed (Fig. 4C).

Mitochondrial free radicals, produced as byproducts during energy metabolism, critically play a role in the pathogenesis of chronic fatigue[31]. Repetitive and intense exercise leads to excess generation of ROS, and it accelerates the oxidative damage in the skeletal muscle Allen et al., 2008[32]. End products of oxidative stress, MDA, was over-produced in the chronic forced exercise group, whereas Ginseng Rh2+ treatment completely normalized the MDA level (Fig. 3). Ginseng Rh2+ has similar effects to NAC in the overall results; however, ginseng Rh2+ is superior in the exercise ability.

Taken together, these findings strongly evidenced that Ginseng Rh2+ exerts potent anti-fatigue properties by modulation of energy homeostasis and oxidative stress. However further studies are required to compare the anti-fatigue effects of Ginseng Rh2+ and general red ginseng using animal models as well

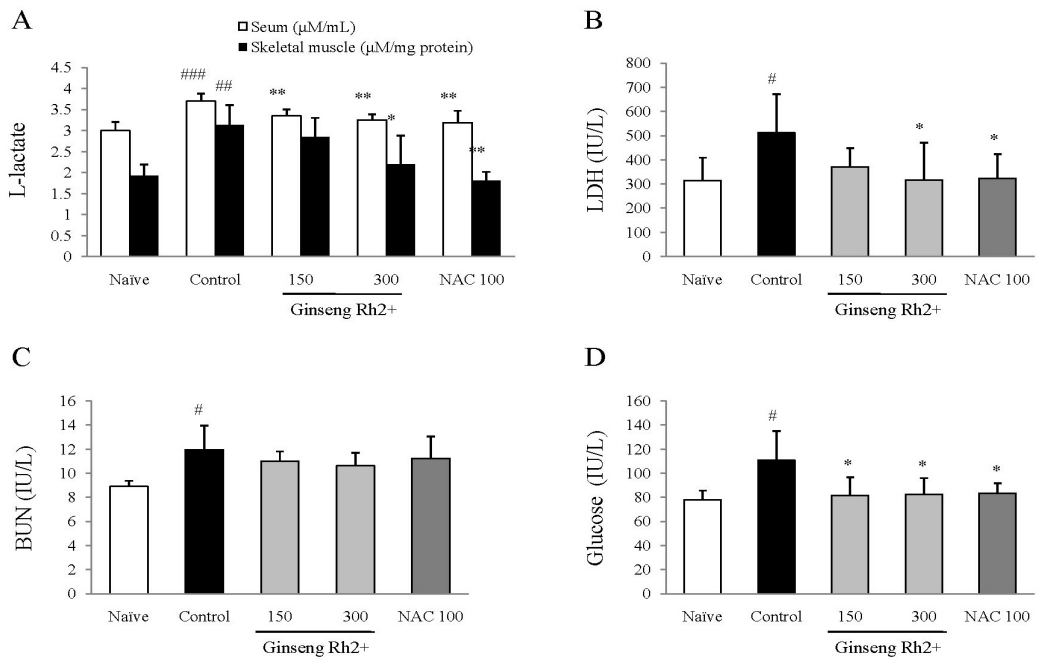

Fig. 4. Changes in skeletal muscle and/or serum L-lactate, LDH, BUN, and glucose levels.
Skeletal muscle and serum levels of l-lactate (A) were determined by ELISA. Serum levels of LDH (B), BUN (C), and glucose (D) were determined using an autoanalyzer. Data are expressed as means ± SD (n = 8). $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ compared with the naïve group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control group.

as clinical trials.

Conflicts of interest

The authors have no conflicts of interest to declare. All of the authors have approved the final article.

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