Anti-fatigue Activity of *Hovenia dulcis* on a Swimming Mouse Model through the Inhibition of Stress Hormone Expression and Antioxidation

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Abstract: *Hovenia dulcis* (H. dulcis) Thumb., which is distributed in Korea, China, and Japan, has been known to show hepatoprotective and free radical scavenging effects and enhance physical activity. Therefore, the objectives of this present study were to determine the anti-fatigue activity of hot-water extract from *H. dulcis* peduncle, and to find the reason why *H. dulcis* extract (HDE)-ingested mice had enhanced physical activity against swimming performance. The mice orally administrated with HDE (HDE-mice) dramatically enhanced their swimming time compared to the control mice. HDE significantly decreased serum levels of stress hormones, such as cortisol and adrenocorticotropic hormone (ACTH) in mice. The levels of thiobarbituric acid reactive substances (TBARS) were dramatically decreased in gastrocnemius muscle from both 100 mg/kg of HDE (LHDE) and 200 mg/kg of HDE (HHDE)-ingested mice compared to the control mice. The liver activities of superoxide dismutase (SOD) were significantly increased in HHDE-mice with increasing tendency in LHDE-mice. In addition, HHDE-mice significantly decreased the levels of blood glucose, total cholesterol (T-Chol), and triglyceride (TG). These results suggest that HDE had a significant anti-fatigue effect via its anti-stress and antioxidant activities, and thereby enhanced physical activity in swimming performance.

**Keywords:** *Hovenia dulcis* Thumb. Extract; Exercise; Anti-fatigue; Physical Activity; Anti-Stress; Antioxidant.

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Introduction

Fatigue is characterized as physical or mental weariness resulting in negative impacts on work performance and exercise intensity, family life, and social relationships (Mehta and Agnew, 2012). Despite the prevalence of fatigue in developed countries, the pathophysiology and etiology of fatigue are unknown (Cho et al., 2010). Fatigue can be classified as secondary, physiologic, or chronic. Secondary fatigue results from disturbed sleep, depression, excess exertion, and medication side effects. Physiological fatigue is caused by inadequate rest, physical effort or mental strain (Brown and Schutte, 2006). Chronic fatigue syndrome involves a persistent unexplainable fatigue lasting for more than six months (Fukuda et al., 1994). Long-term physical and mental fatigue leads to health damage and chronic fatigue (Lowry and Pakenham, 2008). Physical fatigue is called peripheral fatigue and may be accompanied by deterioration in performance (Fitts, 1994). Two mechanisms, oxidative stress and exhaustion, play an important role in physical fatigue (Coombes et al., 2002). Hard work or intense exercise can lead to the production and accumulation of excess reactive free radicals, which results in oxidation stress injury to the body. Exhaustion theory suggests that energy source depletion and excess metabolite accumulation lead to fatigue (You et al., 2011). Several studies, however, have shown that exogenous antioxidants can reduce exercise-induced oxidative stress (Zheng et al., 2012).

Various agricultural products and foods have been investigated as an important resource for postponing fatigue, accelerating the elimination of fatigue-related biomarkers, and improving energy supply, like fermented rice bran, garlic, branched chain amino acids, or medium-chain fatty acids (Kim et al., 2002; Morihara et al., 2007; Hsu et al., 2011; Liu et al., 2011). *Hovenia dulcis* Thunb., which is distributed in Korea, China, and Japan, has been reported to reduce blood sugar and may be an effective anti-diabetic herb (Ji et al., 2002). In addition, *H. dulcis* decreased blood alcohol concentration and removed excessive free radicals caused by drinking alcohol, thereby reducing alcohol-induced liver injury (Wang et al., 2012; Xiang et al., 2012). The fruit and red peduncle are used as a febrifuge and to treat parasitic infections (Buono et al., 2008). The stem bark of this plant has been introduced as a natural drug for the treatment of rectal diseases (Hyun et al., 2010). Recent research indicates that *H. dulcis* contains an extensive variety of pharmaceutically active compounds, such as triterpenoids, flavonoids, and alkaloids. Interestingly, a triterpenoid-rich extract from *Antrodia camphorata* improved physical fatigue and exercise performance in mice (Huang et al., 2012). Interestingly, several studies revealed that *H. dulcis* also had an antioxidant activity in vitro (Li et al., 2005; Wang et al., 2012). Also, we reported that HDE enhanced physical activity of ICR mice in swimming exercise (accepted to Yakhakhhoeji). However, it has not been investigated whether *H. dulcis* has an anti-fatigue effects.

Therefore, the present study investigated the effects of *H. dulcis* hot-water extract on anti-fatigue by evaluating blood stress hormone, such as cortisol and adrenocorticotropic hormone (ACTH), and criteria associated with anti-oxidation in tissue, such as TBARS and SOD activity using swimming mice.
Materials and Methods

Test Substance

*H. dulcis* Thunb. was extracted with 10 volumes of water at 95°C for 4 h and converted to a powder using the spray-dry method. Quercetin was analyzed as a marker compound to confirm that the qualities of HDE were consistent. Quercetin was separated on a Tosoh ODS column (4.6 x 150 mm, 5 μm) by an Agilent Technologies 1200 Series HPLC system (Model Agilent Technologies 1200 Series DAD SL G1315C, USA) and the peak of quercetin was detected at 254 nm. The concentration of quercetin in HDE was 7.6 μg/g. The measurement was performed at the Korea Health Supplement Institute (Bundang, Gyeonggi, Korea).

HDE was dissolved in a sterile water vehicle. The vehicle control group was treated with sterile water alone. All samples were freshly prepared immediately before administration and were administrated at a volume of 10 mL/kg of body weight per mouse.

Animal and Experimental Design

Six-week-old male Slc:ICR mice (Japan SLC, Inc., Japan) were housed individually in Polysulfone cages and identified by animal numbers on their tails. All animals were given a standard laboratory diet (Rodent diet 2918C; Harlan Teklad, Indianapolis, IN, USA) and distilled water ad libitum, and housed at temperature of the animal room (23 ± 1°C) and the humidity ranged from 50–60% with a 12 h light/12 h dark cycle (lights on from 7:00 AM to 7:00 PM). All animal experiments conformed to the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Biotoxtech Co., Ltd. This study was approved by the IACUC ethics committee. Mice were separated into three groups (n = 8 per group in each test) for treatment: (1) vehicle control; (2) 100 mg/kg HDE (LHDE); and (3) 200 mg/kg HDE (HHDE). Vehicle or HDE was administrated by oral gavage. The control group received the vehicle, distilled water, at the same dosage volume of 10 mL/kg throughout the same period.

Animal Observation

All animals were observed once daily for morbidity and third daily for mortality. Body weights were recorded once before the start of the experiment, weekly thereafter, and on the day of study termination.

Forced Swimming

Mice were pre-treated with vehicle, LHDE, and HHDE for three weeks continuously and one hour after the last administration, underwent a swimming test. The swimming performance protocol was adapted from a previous study with some modifications (Porsolt *et al.*, 1977). In brief, the mice were individually carried out in an acrylic plastic pool
(50 W × 40 D × 50 H cm) with 35-cm water depth maintained at 25 ± 2°C. The mouse was taken out from each treatment for swimming exercise and loaded the constant weight (attached to the tail) corresponding to 4% of individual body weight. At the end of the three-week adaptation and dosing period, mice were fasted overnight, and then were forced to swim in the water.

**Determination of Blood Biochemical Parameters**

The effects of HDE on plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), Creatinine (Crea), total cholesterol (T-Chol), Triglyceride (TG), lactate dehydrogenase (LDH), cortisol and adrenocorticotropic hormone (ACTH) were evaluated after exercise. After 1 h of the last administration, a 15-min swimming test was performed without weight-loading. Blood samples were immediately collected from the submandibular duct of pretreated mice after the 15 min swimming test. After 1 h of swimming, mice were anaesthetized with isoflurane and then blood were collected from vena cava. The plasma was prepared by centrifugation at 1000 × g, 4°C for 15 min. ALT, AST, BUN, Crea, T-Chol, TG and LDH levels were determined by an auto-analyzer (Hitachi 7170, Hitachi, Tokyo, Japan). Blood glucose was measured using an Onetouch® ultra™ glucometer (LifeScan Inc., Milpitas, CA, USA). Cortisol and ACTH were determined using a commercially available colorimetric assay kit from Endocrine Technologies (Fircrest Street Newark, CA, USA) and from MD Biosciences (Saint Paul, MN, USA) following the manufacturer’s instructions, respectively.

**Body and Organ Weight**

Body weight was recorded before the forced swimming test, and liver and gastrocnemius muscles were removed from anesthetized mice immediately after completion of the forced swimming test.

**Analysis of Antioxidant Activity in Tissues**

Immediately after the blood had been collected, liver and gastrocnemius muscle were quickly dissected out, frozen in liquid nitrogen, and kept at −70°C until analysis for thiobarbituric acid-reactive species (TBARS) and superoxide dismutase (SOD) activity. TBARS levels and SOD activities were determined using a commercially available colorimetric assay kit from Cell Biolabs (San Diego, CA, USA) following the manufacturer’s instructions.

**Statistical Analysis**

Data are expressed as the mean ± S.D. Comparisons between groups were made using Student’s t-test. p < 0.05 was considered statistically significant.
Results

Body Weight, Muscle and Liver Weight

Morphological data from each experimental group are summarized in Table 1. Before and after the administration of HDE for three weeks, changes of body weight in mice were determined. The body weight did not differ across groups at day 0 or 21 ($p > 0.05$). In addition, there were no significant changes in skeletal muscle mass (gastrocnemius muscle) and liver weights among vehicle, LHDE and HHDE groups, and thus, the short-term supplementation with HDE treatments would not affect the body growth or enhancement of the weight of skeletal muscle.

To further examine whether HDE treatment could cause any negative effect on skeletal muscle tissues of healthy mice, we examined hepatic and muscular morphology in HDE-treated mice. As shown in Fig. 1, no indications of a deleterious effect from HDE treatments were found.

Effect of *H. dulcis* Peduncle Extract on the Clinical Biochemistry Tests

The clinical biochemistry values were measured at the end of experiment in vehicle control and mice treated with LHDE and HHDE. As shown in the Table 2, there were no significant changes in the liver profile (ALT and AST) and renal profile (BUN and Crea), with decreasing tendency with HHDE in cardiac profile (LDH), among vehicle, LHDE and HHDE groups. Interestingly, lipid levels (T-Chol and TG) were significantly decreased to $122.8 \pm 25.8$ and $38.5 \pm 19.4$, respectively, in HHDE ($p = 0.0341$ and 0.0149, respectively), not in LHDE, compared to vehicle control ($163.5 \pm 31.1$ and $67.3 \pm 13.1$, respectively).

Effect of *H. dulcis* Peduncle Extract on the Level of Blood Glucose

Energy storage and supply is another important factor related to exercise performance. In terms of energy expenditure with exercise, rapid ATP consumption and energy deficiency determines the level of physiological fatigue (Sahlin *et al.*, 1998; Belluardo *et al.*, 2001). Thus, blood glucose levels of vehicle control, LHDE and HHDE were $80.2 \pm 32.2$, $45.6 \pm 9.1$ and $40.6 \pm 10.2$, respectively, when those were measured as an energy source in blood samples which were collected after 1 h of swimming, (Fig. 2). Statistical significance was found in HHDE ($p = 0.0489$), not LHDE ($p = 0.0727$). This result suggested that HDE induced better consumption of glucose as an energy source required for exercise in a dose-dependent manner.

<table>
<thead>
<tr>
<th>Table 1. General Characteristics of the Experimental Groups</th>
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<tr>
<td>Three Weeks</td>
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<tr>
<td>Body Weight (g)</td>
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<td>Liver (g%)</td>
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<td>Muscle (g%)</td>
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Figure 1. Effect of HDE treatment on the morphology of skeletal muscle (A) and liver (B). Mice were pretreated with vehicle, 100 (LHDE), and 200 mg/kg (HHDE) of HDE for three weeks, then 1 h after the last treatment, performed 1 h swimming test with weight-load attached to the mouse tail. All mice were sacrificed and examined for the morphology of skeletal muscle and live at the end of experiment. Specimens were photographed with a light microscope (Olympus BX51, Olympus Co. Ltd., Tokyo, Japan). (H&E stain, magnification: ×200, Scale bar, 100 μm).

Effect of H. dulcis Peduncle Extract on the Levels of Blood Stress Hormones

Chronically high cortisol levels ultimately cause a variety of physiological problems, including fatigue. As shown in Fig. 3A, blood cortisol levels were significantly reduced in both LHDE (13.1 ± 2.8, p = 0.0212) and HHDE (12.7 ± 4.4, p = 0.0253) after 1 h of swimming than vehicle control (19.9 ± 5.0). The production of cortisol follows within minutes the ACTH circadian secretion. Thus, we confirmed that the reduced level of blood

<table>
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<tr>
<th>Three Weeks</th>
<th>Vehicle</th>
<th>LHDE</th>
<th>HHDE</th>
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<tbody>
<tr>
<td>ALT (U/L)</td>
<td>25 ± 4.7</td>
<td>25.8 ± 5.3</td>
<td>36.3 ± 24.3</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>46.4 ± 6.6</td>
<td>51.8 ± 7.6</td>
<td>72.3 ± 54.8</td>
</tr>
<tr>
<td>BUN (mg/dL)</td>
<td>24.9 ± 5.1</td>
<td>26.1 ± 3.6</td>
<td>26.2 ± 6.2</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.3 ± 0.0</td>
<td>0.3 ± 0.0</td>
<td>0.3 ± 0.0</td>
</tr>
<tr>
<td>T-Chol (mg/dL)</td>
<td>163.5 ± 31.1</td>
<td>152.8 ± 23.8</td>
<td>122.8 ± 25.8*</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>67.3 ± 13.1</td>
<td>80.0 ± 4.9</td>
<td>38.5 ± 19.4*</td>
</tr>
<tr>
<td>LDH (U/L)</td>
<td>232.3 ± 22.3</td>
<td>238.0 ± 41.7</td>
<td>205.6 ± 64.1</td>
</tr>
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cortisol by HDE inhibited the secretion of ACTH within blood with the same blood samples. As expected, blood ACTH levels were also significantly decreased in both LHDE (96.5 ± 16.1, \( p = 0.0319 \)) and HHDE (99.7 ± 22.4, \( p = 0.0429 \)) than vehicle control (144.2 ± 34.3) (Fig. 3B).

**Effect of *H. dulcis* Peduncle Extract on Antioxidant Activities in Tissues**

To investigate whether administration of HDE to animals before the exercise affects the lipid peroxidation index in the tissues, we assessed the levels of TBARS in the red portion of the gastrocnemius muscle after 1 h of swimming. TBARS levels were significantly decreased in LHDE (7.6 ± 2.0, \( p = 0.0278 \)) and HHDE (6.9 ± 5.2, \( p = 0.0263 \)) than...
Figure 4. Effect of HDE on TBARS levels (A) and SOD activity (B) in tissues after swimming exercise. Mice were pretreated with vehicle, 100 (LHDE), and 200 mg/kg (HHDE) of HDE for three weeks, then 1h later performed 1h swimming test with weight-load attached to the mouse tail. All mice were sacrificed and analyzed for TBARS levels in gastrocnemius muscle and SOD activity in liver. Data represent mean ± S.D. of eight mice in each group. *p < 0.05 compared to vehicle control.

vehicle control (61.7 ± 43.2) (Fig. 4A). And then we assessed the activity of SOD in the liver after 1 h of swimming to identify antioxidant capacity. SOD activity was significantly increased in HHDE (33.8 ± 12.3, p = 0.0167) although it was revealed by increasing amount in LHDE (28.3 ± 8.8, p = 0.0648) than vehicle control (12.2 ± 8.4) (Fig. 4B).

Discussion

Fatigue in response to exercise can be caused by mental disorders, organic central nervous system (CNS) abnormalities, or by peripheral nervous system (PNS) dysfunction or skeletal muscle disease (Rondelli et al., 2009; Boyas and Guevel, 2011). Factors that contribute to feeling tired include neurological and non-neurological causes (Spruit et al., 2005). Exercise-induced muscle fatigue is defined as a reversible loss of muscle force during work over time (Gosker and Schols, 2008; Rainoldi et al., 2008). Decrease in force production during exercise can be regarded as a safety mechanism. If fatigue would not occur or was delayed, structural damage to muscle cells and supportive tissues would occur during the workout.

In this study, we compared the fatigue-alleviating effects of two doses of H. dulcis and vehicle control in exercised and weight-loading mice to identify whether HDE has a functional activity against induced fatigue. Hot-water extract of H. dulcis peduncle dose-dependently enhanced exercise performance and reduced muscle fatigue physiological indexes. So, H. dulcis may have ergogenic and anti-fatigue functions.

The energy metabolism of muscular activity determines the level of physiological fatigue (Belluardo et al., 2001). Exercise endurance is an important variable in evaluating anti-fatigue treatment. In physical exercise swimming test, maximal swimming time was increased dose-dependently with HDE doses (accepted to Yakhakhoeji). Glucose, one of the biochemical parameters, is an important indicator of muscle fatigue after exercise (Brancaccio et al., 2007). In terms of energy expenditure with exercise, rapid ATP
consumption and energy deficiency are critical causes of physical fatigue (Sahlin et al., 1998). Glucose is the predominant source of glycolysis for ATP production. Therefore, blood glucose levels directly affects exercise ability. Figure 2 implies the HDE effectively uses glucose as an energy source with statistical significance after exercise.

Responses to stressful events are generally regarded as reactions of the organism to accommodate to or compensate for stress. This reaction is classically described as an activation of the sympathoadrenal system and the hypothalamic-pituitary-adrenocortical (HPA) axis. Activation of the release of stress hormone such as cortisol and ACTH in blood occurs during various types of stress, including exercise (Jankord et al., 2009; Ciocci et al., 2011; Catoire et al., 2012). Therefore, excess swimming led to high levels of stress hormones, and HDE treatment decreased them (Fig. 3). In addition, the primary functions of cortisol are to increase blood sugar through gluconeogenesis from certain amino acids, glycerol, lactate and/or propionate and through glycolysis, which is facilitated by the activation of glycogen phosphorylase (Li et al., 2002; Jellyman et al., 2012). Moreover, in the case of exercise, cortisol secretion causes to increase of blood levels of T-Chol and TG. Reduction of blood cortisol level by HDE treatment seems to lower the level of blood glucose (Figs. 2 and 3A) and the level of T-Chol and TG (Table 2).

Exercise always induces the generation of ATP with the production of superoxide anion. Oxidative stress is the status of deterioration of the balance between oxidant formation and antioxidant defense, which leads to an increase in the amount of antioxidants (Urso and Clarkson, 2003). An increase in TBARS reflects lipid peroxidation by free radicals, indirectly indicating oxidative stress-induced cellular membrane damage. TBARS is commonly used to measure systemic oxidative stress. Many factors contribute to TBARS levels. For example, fasting (Pirjsi et al., 2006) and the post-prandial state (Ursini et al., 1998), which decreases in the former condition because antioxidant activity increases and is elevated in the latter condition, reflecting the peroxide content of a meal. In our study, despite no significant changes in body weight, TBARS levels were significantly decreased in HDE treatment, implying that the reduction of TBARS levels was not caused by other factors except HDE (Fig. 4A). And also decreased SOD activity indicates that the body’s antioxidant system was depleted, whereas an increase indicates that the antioxidant system is very active and strong enough to effectively remove superoxide anion (Wang et al., 2008). Increased SOD activity by HDE implies that harmful oxygen radicals induced by exercise are converted into hydrogen peroxide, which is less harmful in the body (Fig. 4B).

In conclusion, the present study suggest that HDE has an effect of anti-fatigue against swimming performance through better production of physical energy and reduction of stress hormone and oxidation of tissue.

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References


