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Hot Water Extracts of Leather Carp and SoftShell Turtle Improve Exercise Performance in Mice

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(향어 *Cyprinus carpio nudus*와 자라 *Trionyx sinensis* 열수추출물 투여 생쥐의 운동 수행능력 향상)

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A dissertation

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Abstract

The hot water extract of leather carp *(Cyprinus carpio nudus)* and freshwater softshell turtle *(Trionyx sinensis)* extract have been used traditionally as a nourishing tonic soup, and to recover from physical fatigue. In this study, we investigated the effect of that extracts on exercise performance in mice. Swimming endurance and forelimb grip strength were assessed following oral administration of the extract (once per day for 7 days) at a dose of 0.5 mg/10 uL/g body weight. After 7 days, mice given the leather carp extract had significantly greater swimming endurance [105±18 s ($P < 0.05$); 52% longer than day 0]. T. sinensis extract significantly increased the grip strength to 1.25±0.07 N ($P < 0.01$), which is 16.8% h igher than the force on day 0. The extract increased muscle mass, but had little effect on body. After exercising, folle dismutase levels in leater carp extract-fed mice were significantly higher (145%, 131%, and 106%, respectively) than in the saline control owing the swimming exercise, blood

glucose, glutathione peroxidase, and superoxidgroup. Bloodlevels of high-density lipoprotein cholesterol were also significantly increased (128%) in mice given the extract compared to the controls. In T. sinensis extract group, the blood glucose levels were 202% higher and urea levels were 73% lower, which were both significantly different than the levels observed after control treatment. Lactate dehydrogenase was significantly higher by 314%, and glutathione peroxidase increased by 165%. In addition, the obesity markers, serum triglyceride and cholesterol, decreased to 62% and 49%, respectively, after mice were fed the extract. These results suggest that leather carp and T. sinensis extracts can improves physical exercise performance and decreased the oxidative stress caused by an exhaustive workout.

Chapter 1

Hot water extracts of leather carp *Cyprinus carpio nudus* improves exercise performance in mice

1.1. Introduction

Leather carp *Cyprinus carpio nudus* (Linnaeus), known as Israeli carp, German carp, or fragrant fish in Korea, is a part of the Cyprinidae family. Leather carps have large and thick scales distributed along the lower dorsal fin, but lack scales on the rest of the body. They inhabit warm, deep, slow-flowing, and still waters such as lowland rivers and vegetated lakes. They grow fast and have a strong appetite, feeding on a variety of benthic organisms and plant material. The flesh has a firm, chewy texture, with no fine bones or earthy musty odor (1). Thus, leather carp has become popular as a raw flesh or as a hot chowder food. Additionally, the hot water extract of leather carp has been used as a nourishing tonic soup and as an aid for recovery from physical fatigue (2). Leather carp is commercially important in the inland aquaculture industry as one of the major food fish species. In 2013, the production of leather carp by inland water fisheries in Korea was 1,068 metric tons (wet weight), worth $7.4 million US dollars (3).

The effectiveness of various natural food products in improving exercise performance is of interest to sport and healthcare businesses. During physical exercise, the contracting muscles generate force/power, metabolites, and heat, which ultimately induce fatigue and exhaustion (4). Fatigue is categorized as physical or emotional exhaustion resulting in negative effects on physical endurance capacity, work performance, and exercise intensity. Hard work or intense exercise can lead to the production and accumulation of excess reactive oxygen species (ROS), increasing the level of oxidative stress in the body (5). Extracts from a variety of natural food sources have been studied as potential exercise supplements to help improve performance and recover from physical fatigue (6, 7). Most active compounds in exercise supplements, such as peptides (8), polysaccharides (9–11), flavonoids (12), and terpenoids (13), originate from herbal sources. Recently, compounds from animal sources, such as antioxidant

peptides from grass carp *Ctenopharyngodon idellus* (14) and pig spleen (15), have been identified as enhancers of swimming endurance. To date, few studies have examined compounds from aquatic organisms as potential exercise performance enhancers. To investigate the health and anti-fatigue claims related to leather carp extract, we evaluated its effect on swimming endurance, forelimb grip strength, and blood biochemical factors in mice.

1.2. Materials and Methods

1.2.1. Extract preparation and reagents

Fresh leather carp *Cyprinus carpio nudus* (Linnaeus) fillets were obtained from the Kumgu Aquaculture Farm (Kimje, Jeonbuk, Korea), and a voucher specimen is kept in the author's laboratory (M.R. Kim). Fillets (2 kg) were extracted with boiling water (20 L) for 25 h. The residue and oil layer were removed by centrifugation at 3,000 *g* for 10 min, and the aqueous portion was concentrated using a rotary evaporator to obtain the extract (327 g). The extract was adjusted to 50 mg/mL (or 30 mg/mL as protein) with distilled water. Bovine serum albumin was used as the standard for determining protein content (16). Assay kits for determining glucose (AM201-K), urea (AM165-K), glutamic oxaloacetic transaminase (GOT; AM101-K), glutamic pyruvic transaminase (GPT; AM101-K), triglyceride (AM157S-K), high-density lipoprotein (HDL) cholesterol (AM203-K), and total cholesterol (AM202-K) were purchased from Asan Pharmaceutical (Seoul, Korea). The lactate (K627-100), glutathione peroxidase (GPx; K762-100), and superoxide dismutase (SOD; K335-100) kits were purchased from BioVision (Milpitas, CA, USA). The other reagents used were of analytical grade and purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

1.2.2. Swimming endurance test

ICR mice (6–8 weeks old) weighing 23–27 g were purchased from Hyochang Science (Daegu, Korea). Mice were kept in a controlled environment at 24 ± 1°C under a 12-h light/12 h dark cycle at 65% humidity, with a maximum of 5 animals per cage. Feeding consisted of standard animal pellets (Formula™ M07;FeedLab,Guri,Korea)andwater*ad libitum*. Mice were treated in compliance with current laws and guiding principles for the care and use of laboratory animals approved by the Animal Ethics Committee of Pukyong National University (Busan, Korea). The ethics committee approved this study under protocol AEC-201405. The mice (n = 12 per group per test) were orally administered the extract once per day for 7 days at a dose of 10 μL/g body weight (17). One hour after each administration, mice underwent body weight and swimming time measurements. Pure octacosanol (Ferngrove Pharmaceuticals Pty, Ltd., South Granville, Australia) was used as a positive control using an oral dose of 6.7 ng/10 μL/g body weight as per the supplier's protocol. The control group was orally administered saline. For the swimming endurance test, mice were placed individually in a 1-L beaker (25-cm height, 10-cm diameter) filled with water (10 cm deep) at 24 ± 1°C. The total duration of immobility, after a delay of 2 min, was measured for a period of 4 min (6). Each mouse was considered to be immobile when it ceased struggling and remained floating motionless in the water, making only movements necessary to keep its head above water. During the 4-min swim test, the duration of swimming time was calculated by subtracting the total immobilized time. Swimming endurance (sec) was measured three times: before treatment (day 0); 1 h after administrating treatment on the third day (day

3); and 1 h after administrating treatment on the seventh day (day 7).

1.2.3. Forelimb grip strength

Mice (n = 12 per group per test) were orally administered hot water extract, octacosanol (positive control), or saline (negative control) as described above. One hour after each administration, mice underwent forelimb grip strength measurements. A low-force testing system (Model-RX-10, Aikoh Engineering Co., East Osaka, Japan) was used to measure grip strength. The amount of tensile force generated by each mouse was measured using a force transducer with a rectangular 4 × 5-cm2 metal net (18). The mouse was grasped at the base of the tail and pulled slightly backward by the tail while the two forelimbs gripped the metal net. This caused a counter-pull, and the grasping force was recorded by the grip strength in Newton (N). Prior to the experiment, mice were trained to perform this procedure for 3 days. Grip strength (N) was measured 3 times: before treatment (day 0); 1 h after treatment on the third day (day 3); and 1 h after the seventh treatment (day 7). Each mouse was subjected to 3 grip trials with a 1-min rest between trials. The maximum force exerted by the mouse counter-pull was recorded as the forelimb grip strength.

1.2.4. Muscle mass

Mice (*n* = 12 per group per test) were orally administered leather carp extract, octacosanol (positive control), or saline (negative control) as described above. One hour after the last administration, mice were humanely euthanized by cervical dislocation. After peeling the skin off, forelimb and hindlimb were disarticulated from scapula to carpus and from ilium to medial malleolus, respectively, in a consistent manner. They were quickly weighed and the volume

was measured in a 10 mL syringe. The volume was given with muscle and bone. After boiling for 1 min to enable muscles to be cleaned easily, only the bone was weighed. Muscle mass of forelimbs and hindlimbs were calculated using lean mass excluding bone mass.

1.2.5. Biochemical assays

Thirty min after the final swimming trial, blood samples from each mouse were collected. Mice were anesthetized with Zoletil 50 (Virbac, Carros, France; 10 mg/kg, i.m.), and blood was drawn using the facial vein technique (19). Blood was allowed to clot for 10 minutes, followed by centrifugation at 3,000 *g* for 15 min to obtain serum. Using a UV/Vis spectrophotometer (Optizen 2120UV, Mecasys Co., Daejun, Korea), serum levels of glucose, lactate, urea, GPx, SOD, GOT, GPT, triglyceride, HDL cholesterol, and total cholesterol were determined by glucose oxidase, lactate dehydrogenase, urease-indophenol, GPx colorimetric assay, SOD colorimetric assay, Reitman–Frankel method, Reitman–Frankel method, lipoprotein lipase, HDL cholestase, and cholesterol esterase-oxidase methods, respectively.

1.2.6. Statistical analysis

Statistical analysis was performed using the Student's *t*-test. All of the animal experiments were done with a minimum of 12 mice per group. Data are reported as the mean ± standard error (SE).

1.3. Results and Discussions

In a comparison test evaluating the ability of various fish extracts to enhance

exercise capacity, leather carp had the most potent enhancing effect on swimming time among seven common freshwater fish species (bagrid catfish, channel catfish, freshwater eel, leather carp, marsh snail, softshell turtle and trout; data not shown). Given this finding, the effect of leather carp extract on exercise performance was further investigated. Following oral administration of leather carp extract in mice, swimming time averaged 82 ± 13 sec and 105 ± 18 sec ($P < 0.05$) on days 3 and 7, respectively (Fig. 1); this represents increases of 19% and 52%, respectively, compared with the swimming time on day 0 (69 ± 13 sec). Swimming capacity increased significantly with the number of days the mice were fed. On day 7, swimming time was 3-fold longer in the leather carp group than in the saline control group. Octacosanol treatment, acting as a positive control, marginally increased swimming endurance by 8% and 15% on days 3 and 7, respectively. Similarly, increases of 8% (day 3) and 17% (day 7) were observed in the saline group. Our data demonstrate that leather carp extract has a much stronger enhancing effect on swimming endurance than does octacosanol or saline. The forelimb grip strength of mice given the leather carp extract averaged 1.08 ± 0.05 N ($P < 0.05$) and 1.18 ± 0.05 N ($P < 0.01$) on days 3 and 7, respectively (Fig. 2); this represents increases of 7% and 17%, respectively, compared with the grip strength on day 0 (1.01 ± 0.03 N). Grip strength increased significantly with the number of days the mice were fed. The increase in grip strength observed on day 7 was 19-fold stronger in the leather carp group than in the saline control group. Octacosanol treatment increased grip strength by 8% and 11% on days 3 and 7, respectively. Grip strength in the saline group changed by –1% (day 3) and –2% (day 7). Our data show that leather carp extract and octacosanol have similar effects on grip strength in mice. Body weights of mice given the leather carp extract averaged 24.8 ± 1.0 g on days 3 and 7 (Fig. 3), which represents a 2% increase compared with day 0 (24.3 ± 1.1 g). In mice given octacosanol, body weight increased by 8% ($P < 0.05$) and 12%

($P < 0.01$) on days 3 and 7, respectively, compared with day 0. Thus, octacosanol treatment had a greater effect on body weight than the leather carp extract. No change in body weight was observed in mice given saline. Overall, the leather carp extract significantly increased swimming capacity and grip strength, but had little effect on body weight. Forelimb muscle mass of mice given the leather carp extract averaged 0.76 ± 0.02 g ($P < 0.01$) on day 7 (Fig. 4), which represents a 30% increase compared with saline (0.59 ± 0.03 g). In mice given octacosanol, forelimb muscle increased by 46% compared with saline. Hindlimb muscle mass of mice given the leather carp extract averaged 1.47 ± 0.06 g ($P < 0.01$) on day 7, which represents a 20% increase compared with saline (1.22 ± 0.05 g). In mice given octacosanol, hindlimb muscle increased by 24% compared with saline. Thus, the leather carp extract and octacosanol had great effects on muscle mass of forelimbs and hindlimbs. Significant increases in volumes of forelimb and hindlimb were also observed. Overall, the leather carp extract significantly increased muscle weight and volume, but had little effect on total body weight.

To investigate the anti-fatigue or fatigue recovery properties of the leather carp extract, biochemical analyses were performed on blood samples collected 30 minutes after the swimming test on day 7. In mice given the leather carp extract, serum glucose levels averaged 11.2 ± 2.0 mM/L, a 145% increase over saline-treated mice ($P < 0.01$) (Table 1). The levels of lactate and urea were 515.1 ± 11.4 and 1.1 ± 0.0 mM/L, respectively, which were lower than the levels in the control mice. Thus, the extract induced higher levels of blood glucose, an energy source for exercise performance and fatigue recovery. The activities of GPx and SOD, antioxidant enzymes, in the extract-fed mice were 1,240.4 ± 92.8 mU/mL ($P < 0.05$) and 907.3 ± 9.9 mU/mL ($P < 0.01$), respectively, which were significantly higher than those in the control group. Therefore, the leather carp extract decreased oxidative stress caused by an exhaustive workout. Activities of GOT and GPT, measures of liver toxicity, were all within the normal range of 0–

40 IU/L. Serum HDL cholesterol was 4.6 ± 0.4 mM/L, which was significantly higher than in the control group (128%, $P < 0.05$). The triglyceride and total cholesterol levels stayed relatively constant. Even after 7 days of leather carp extract exposure, mice did not accumulate triglyceride or total cholesterol, both of which are markers for obesity at high concentrations.

Leather carps grow fast and are used as a tonic food. Aquaculture of leather carp has become popular in freshwater farms and ponds. Ingestion of raw carp bile is known to induce acute hepatotoxicity and nephrotoxicity in humans (20). The flesh fillet or whole body, after removing the gall bladder, is generally used for cooking. Leather carp flesh has a relatively high amount of lysine (3.5 g/100 g wet tissue) (21), which is a limiting essential amino acid in major plant-food protein sources (22). Leather carps have high nutritional value, especially for plant-food consumers. Additionally, lysine is considered to have better antioxidant properties (23), although all 20 of the amino acids found in proteins have the potential to interact with free radicals (24). Antioxidant mechanisms of many proteins are dependent on amino acid composition. *In vitro* radical scavenging activities of leather carp extract against

2, 2′-azinobis[3-ethylbenzthiazoline]-6-sulfonic acid (ABTS), superoxide, and hydroxyl radicals have ranged from 50 to 60% (25), which is similar to the values obtained with silver carp protein hydrolysate (26) and loach protein hydrolysate (27). The antioxidant properties of leather carp extract allow neutralization of free radicals, which may help in delaying fatigue and limiting oxidative damage of tissues.

Among blood biochemical parameters, the homeostasis of serum glucose level is very important for improving exercise performance. Physical exercise necessitates a higher rate of metabolism to handle the increased energy demands. When hypoglycemia is prolonged, it can overwhelm the glycogen storage and reduce blood glucose (28). Blood glucose levels reflect the grade and speed of

fatigue development. A modest workout begins with an increase in aerobic muscle activity; however, rigorous exercise triggers anaerobic metabolism, leading to a decrease in blood glucose and an accumulation of lactic acid (29). When mice were fed the leather carp extract, blood glucose levels increased to near hyperglycemic levels. It is known that hyperglycemia may change the organization of membrane lipids, which is correlated with increased formation of lipid hydroperoxide (LOOH), an intermediate product of oxidative lipid damage (30). Leather carp extract also increased GPx and SOD antioxidant activities in the blood, which may help protect against oxidative lipid damage. Additionally, we found that mice given the leather carp extract did not show any problematic symptoms of hyperglycemia, such as body weight loss or physical tiredness. Thus, the extract appeared to facilitate the release of glucose from glycogen, allowing the animals to regain energy after a workout. Similar results were obtained when fungus (13) or fenugreek (31) extracts were used to improve exercise performance and decrease physical fatigue. Exhaustive heavy exercise generates ROS (5). The activities of GPx and SOD, major antioxidant enzymes, were used as a measure of the anti-fatigue effect of leather carp extract. GPx and SOD activities in the extract-fed mice were much higher than those in the saline control group. This suggests that the extract decreases oxidative stress by counteracting the oxidative effects of free radicals produced during exhaustive workouts, similar to phenolic compounds (32) and some dietary antioxidants (33). The anti-fatigue capacity of the extract may have also been facilitated by lower levels of ROS, such as LOOH, via increased blood HDL. HDL in the blood acts as an antioxidant by reducing levels of LOOH (34). HDL has been found to increase peripheral glucose uptake through the activation of AMP-activated protein kinase (AMPK) in muscle cells (35) and insulin secretion by pancreatic ß cells (36). This suggests a potential metabolic role for HDL in the modulation of plasma glucose homeostasis, in addition to its well-established role in reverse

cholesterol transport and modulation of inflammation (37). Though human data are still unavailable, it is plausible that the plasma concentration of HDL may be one of the factors that modulate the duration and intensity of transient hyperglycemia. For prevention of cardiovascular diseases and diabetes, many studies have shown that a diet supplemented with fruit and vegetables has beneficial effects for reduction of cholesterol and glucose (38,39). Although the mechanisms are unclear regarding the leather carp extract-induced increases in blood HDL, glucose, GPx, and SOD, the observed enhancements in exercise performance are likely to be complex, possibly involving a combination of bioactive components in the extract. Further research is necessary to investigate the isolated bioactive components. Nevertheless, our study indicates that leather carp extract is beneficial for preventing the oxidative stress caused by exhaustive workouts. Comparing to the octacosanol as a positive control, the leather carp extract reduced blood lactate significantly. The triglyceride/HDL ratio is considered a better predictor of coronary arterial disease than lipid alone, and a stronger predictor of myocardial infarction than classical atherogenic indices (LDL:HDL cholesterol ratio) (40). The triglyceride/HDL ratio in mice given the leather carp extract was 0.48, compared with 0.63 in the saline control group. The ratio in the octacosanol group was 0.53 even though the levels of triglycerides and cholesterol were lower than in the leather carp extract and saline groups. Thus, it is proposed that leather carp extract alleviates, rather than induces, cardiovascular disease and atherosclerotic progression. In summary, our findings indicate that leather carp *C. carpio nudus* extract may be useful in health food products for enhancing physical performance and managing the onset of fatigue.

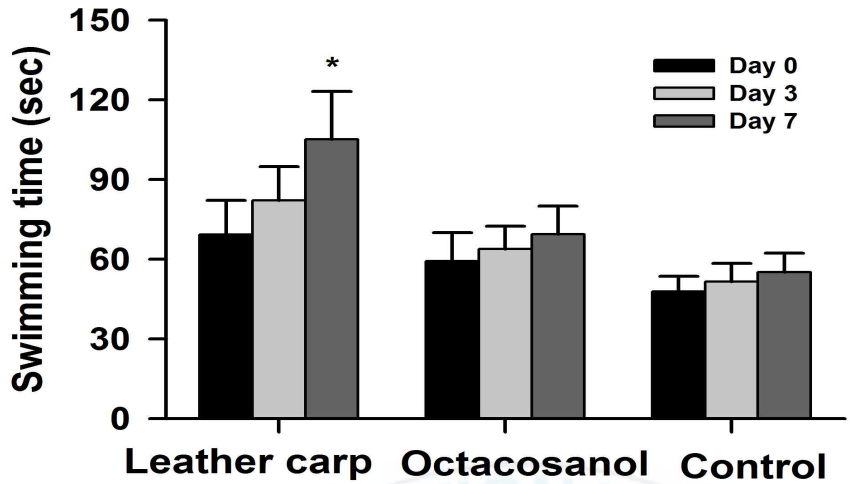

Figure 1. Effect of leather carp extract on swimming endurance. Mice were orally administered the extract once per day for 7 days at a dose of 0.56 mg/10 μL/g body weight, and underwent the swimming test 1 h after administration. Octacosanol, the positive control, was given at a dose of 6.7 ng/10 μL/g body weight. Data are presented as the mean ± SE (n = 12). *P < 0.05 compared with day 0.

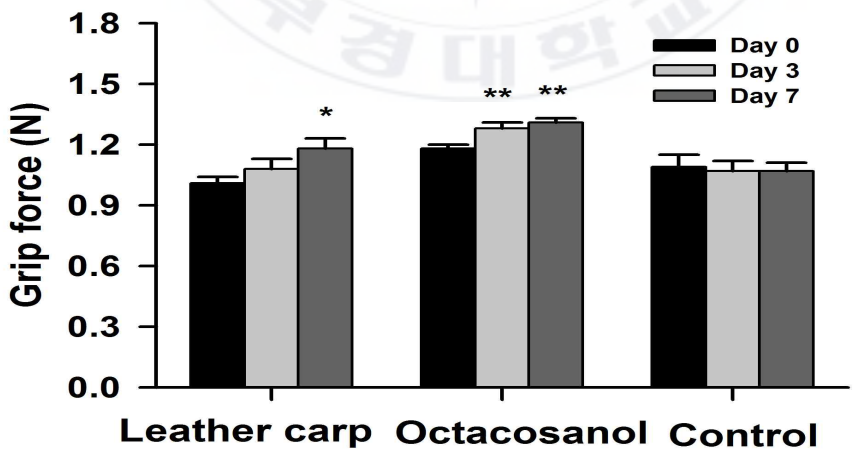

Fig. 2. Effect of leather carp extract on forelimb grip strength. Mice were orally administered the extract once per day for 7 days at a dose of 0.56 mg/10

μL/g body weight, and underwent the grip test 1 h after administration. Octacosanol, the positive control, was given at a dose of 6.7 ng/10 μL/g body weight. Data are presented as the mean ± SE (n = 12). *P < 0.05 and **P < 0.01 compared with day 0.

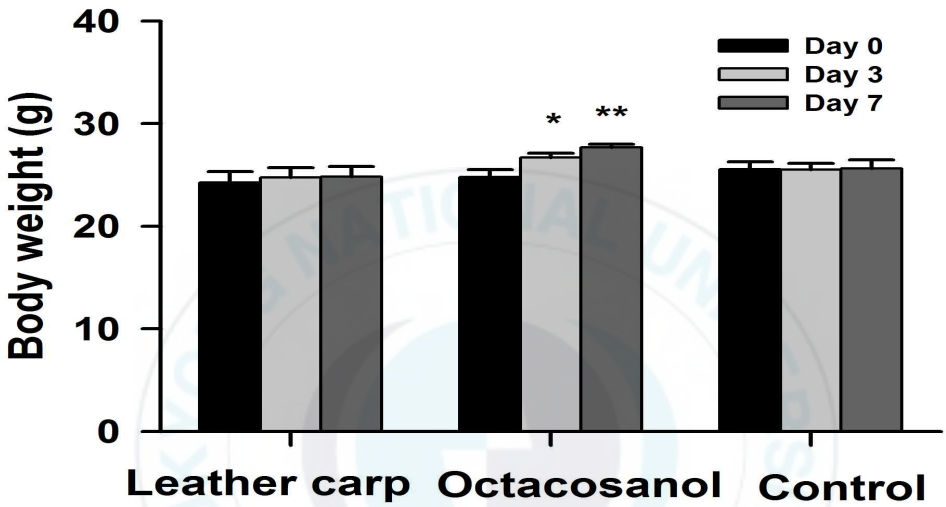

Fig. 3. Effect of leather carp extract on body weight. Mice were orally administered the extract once per day for 7 days at a dose of 0.56 mg/10 μL/g body weight, and body weight was measured 1 h after administration. Octacosanol, the positive control, was given at a dose of 6.7 ng/10 μL/g body weight. Data are presented as the mean ± SE (n = 12). *P < 0.05 and **P < 0.01 compared with day 0.

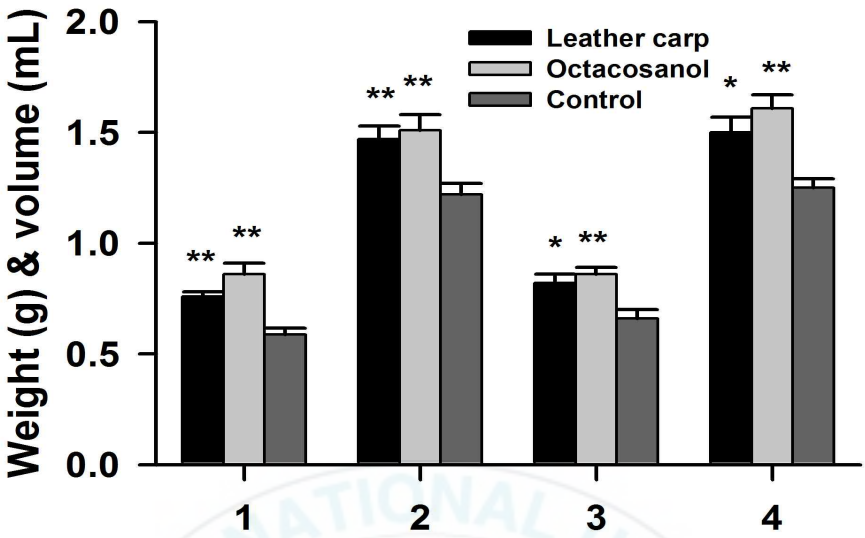

Fig. 4. Effect of leather carp extract on muscle mass of forelimb and hindlimb. Mice were orally administered the extract once per day for 7 days at a dose of 0.56 mg/10 μL/g body weight or the octacosanol at a dose of 6.7 ng/10 μL/g body weight. Weight (1&2) and volume (3&4) of forelimbs (1&3) and hindlimbs (2&4), respectively, were measured 1 h after the final administration. Data are presented as the mean ± SE (n = 12). *P < 0.05 and **P < 0.01 compared to control.

Table 1. Effect of leather carp extract on serum glucose, lactate, urea, GPx activity, SOD activity, GOT activity, GPT activity, triglyceride, HDL cholesterol, and total cholesterol after the swimming test

	Glucose (mmol/L)	Lactate (μmol/mL)	Urea (mmol/L)	GPx (mU/mL)	SOD (mU/mL)	GOT (IU/L)	GPT (IU/L)	Triglyceride (mmol/L)	HDL cholesterol (mmol/L)	Total cholesterol (mmol/L)
Saline	7.7 ± 0.6	545.5 ± 13.7	1.2 ± 0.1	949.8 ± 54.4	856.4 ± 13.9	29.9 ± 3.9	19.1 ± 1.5	2.3 ± 0.1	3.6 ± 0.3	7.8 ± 0.4
Leather carp	11.2 ± 2.0**	515.1 ± 11.4	1.1 ± 0.0	1,240.4 ± 92.8*	907.3 ± 9.9**	23.7 ± 2.3	14.6 ± 2.5	2.2 ± 0.1	4.6 ± 0.4*	8.2 ± 0.2
Relative activity (%)[1)]	145	94	92	131	106	79	76	96	128	105
Octacosanol	12.2 ± 0.8**	822.9 ± 25.5**	0.9 ± 0.0**	1104.0 ± 67.9	1344.3 ± 61.5**	28.6 ± 5.8	24.3 ± 3.2	1.7 ± 0.1**	3.2 ± 0.1	5.7 ± 0.2**
Relative activity (%)[1)]	158	151	75	116	157	96	127	74	89	73

GPx, glutathione peroxidase; SOD, superoxide dismutase; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; HDL, high-density lipoprotein. Values are the means ± SE (n = 12). *P < 0.05 and **P < 0.01 compared to control. [1)]Relative activities are expressed as percentages of the values against the saline control group.

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Chapter 2

Oral Administration of a Hot Water Extract of the Softshell Turtle *(Trionyx sinensis)* Improves Exercise Performance

2.1. Introduction

The effectiveness of various natural food products in improving exercise performance is of interest to sports and healthcare industries. During physical exercise, the contracting muscles generate force or power, metabolites, and heat, which ultimately induce fatigue and exhaustion (1). Fatigue is categorized as physical or emotional exhaustion resulting in negative effects on physical endurance capacity, work performance, and exercise intensity. Hard work or intense exercise can lead to the production and accumulation of excess reactive free radicals, which results in oxidative stress injury to the body (2). Several natural food resources have been studied as supplements to improve exercise performance and recovery from physical fatigue (3-5). Most of these are polysaccharides or terpenoids from herbal sources. Nevertheless, few studies have examined agents from aquatic organisms that can improve exercise performance. Traditionally, the water-soluble extract of the freshwater softshell turtle, Trionyx sinensis, has been used as a tonic soup, and to recover from physical fatigue (6). T. sinensis is the most common turtle species raised in Asia, and has immense aquaculture potential. In 2012, the production of softshell turtles by inland water fisheries in Korea was 250 metric tons (wet weight), worth $12 million US dollars (7). To evaluate the tonic activity of T. sinensis, changes in forelimb grip strength and anti-fatigue activity were measured in mice fed the hot water extract prepared in the traditional way.

2.2. Materials and Methods

2.2.1. Extract preparation and reagents

Fresh softshell turtle (*T. sinensis*) bodies were obtained from the Gunwi Zara Aquaculture Farm (Ubo, Gunwi, Korea). A voucher specimen is deposited in the laboratory of Dr. M. Kim (Silla University, Busan, Korea). Whole bodies (2kg) were extracted with boiling water(20L) for 25 h. After removing the residue and oily layer by centrifugation at 3,000 *g* for 10 min, the aqueous portion was concentrated using a rotary evaporator to obtain the extract (220g;yield11%). The extract was adjusted to 88mg/mL (or 50 mg/mL as protein) with distilled water. Bovine serum albumin was used as the standard when determining the protein content (8). Assay kits for determining glucose (AM201-K), urea (AM165-K), lactate dehydrogenase (LDH; LDH-LQ), triglyceride (AM157S-K), and cholesterol (AM202-K) were purchased from Asan Pharmaceutical (Seoul, Korea). The glutathione peroxidase (GPx) kit (K762-100) was purchased from BioVision (Milpitas, CA, USA). The other reagents used in this study were of analytical grade.

2.2.2. Forelimb grip strength

More than 48 ICR mice (6−8 weeks old) weighing 23−27 g were purchased from Hyochang Science (Daegu, Korea). Mice were kept in a controlled environment at 24 ± 1°C under a 12 h light/12 h dark cycle at 65% humidity, with

a maximum of five animals per cage. The animals were fed standard animal pellets (Formula™ M07;FeedLab,Guri,Korea)andwater*ad libitum*. The mice were treated in compliance with current laws and guiding principles for the care and use of laboratory animals approved by the Animal Ethics Committee of Pukyong National University (Busan, Korea). The ethics committee approved this study under protocol AEC-201405. The mice (n = 12 per group per test) were orally administered the extract once a day for 7 days with each 1 μL/g body weight, and then underwent body weight and grip strength measurements 1 h after each administration. The vehicle group was orally administered saline as a control. A low-force testing system (Model-RX-10, Aikoh Engineering Co., East Osaka, Japan) was used to measure the forelimb grip strength of the mice. The amount of tensile force generated by each mouse was measured using a force transducer with a rectangular 4 × 5 cm^2metalnet(9). The mouse was grasped at the base of the tail and pulled slightly backward by the tail while the two forelimbs gripped the metal net, which caused a counter-pull. The grip strength meter recorded the grasping force in Newtons (N). Before the experiment, the mice were trained to perform this procedure for 3 days. Grip strength (N) and body weight (g) were measured three times: before treatment (day 0); 1 h after treatment on the third day (day 3); and 1 h after the seventh day (day 7). Each mouse was subjected to three grip trials with at least a 1 min rest between trials. The maximum force exerted by the mouse counter-pull was recorded as the forelimb grip strength.

2.2.3. Biochemical assays

Thirty min after the last grip strength trial, blood samples were collected from mice. The mice were anesthetized with Zoletil 50 (Virbac, Carros, France; 10 mg/kg, i.m.), and then blood was drawn using the facial vein technique (10). After clotting the blood for 10 min, it was centrifuged at 3,000 *g* for 15 min to obtain serum. The serum levels of glucose, urea, LDH, GPx, triglyceride, and total cholesterol were determined by glucose oxidase, urease-indophenol, lactate substrate, GPx colorimetric assay, lipoprotein lipase, and cholesterol esterase-oxidase methods, respectively, using an UV/Vis spectrophotometer (Optizen 2120UV, Mecasys Co., Korea).

2.2.4. Statistical analysis

Statistical analysis was performed using the Student's *t*-test. All of the animal experiments were done with at least 12 mice per group. Data are reported as the means ± standard deviation (SD).

2.3. Results and Discussions

The forelimb grip strength of the mice fed the softshell turtle extract averaged 1.19 ± 0.06 N ($P<0.01$) and 1.25 ± 0.07 N ($P<0.01$) on days 3 and 7, respectively (Fig. 1A), which represented an increase of 6.9% and 16.8%, compared to the force on day 0 (1.04 ± 0.06 N). Control treatment increased the strength by −0.2% and 0.3% on days 3 and 7, respectively. Grip strength

significantly increased with the time that the mice were fed the extract. The body weights of mice fed the extract averaged 25.3 ± 1.1 g and 25.6 ± 1.0 g after 3 and 7 days, respectively (Fig. 1B), which were marginally increased by 1.0% and 2.3% compared to day 0 (25.0 ± 1.1 g). With the control treatment, body weight increased by −0.2% and 0.3% after 3 and 7 days, respectively compared to day 0. Therefore, the *T. sinensis* extract significantly increased grip strength, and resulted in a marginal increase in body weight.

To investigate the anti-fatigue or fatigue recovery effects of the *T. sinensis* extract, blood samples for biochemical analysis were collected at 30 min after trials on day 7.

In the extract-fed mice, serum glucose levels averaged 9.9 ± 1.0 mmol/L, which was 202% higher (P<0.01) than that in the control mice. Thus, presence of the extract led to the provision of more blood glucose as energy source for the forelimb exercise and fatigue recovery. In addition, urea level averaged 3.5 ± 0.8 mmol/L, which was 73% lower (P<0.01) than that in control mice (Table 1). The *T. sinensis* extract may prevent the release of blood urea nitrogen via protein catabolism. The activity of LDH in the mice fed the extract averaged 2,275.4 ± 697.3 U/L (P<0.01), which was 314% higher than that of the control group. As an antioxidant enzyme, the activity of GPx in the extract-fed mice was 514.7 ± 153.8 mU/mL (P<0.01), which was 165% higher than that of the control group.

Therefore, the *T. sinensis* extract decreased the oxidative stress caused by an exhaustive workout. Serum triglyceride and total cholesterol levels were 0.8 ± 0.3 mmol/L (P<0.05) and 1.9 ± 0.4 mmol/L (P<0.01), respectively. Even after being fed the softshell turtle extract for 7 days, the mice did not accumulate triglyceride and cholesterol. Rather, the triglyceride and cholesterol levels decreased by 62% and 49%, respectively, upon feeding of the extract. This may be caused by the increased breakdown of triglycerides to free fatty acids which are subsequently oxidized.

Among biochemical parameters, the homeostasis of blood serum glucose is very important for improving exercise performance. Training frequently leads to hypoglycemia, which necessitates a higher rate of metabolism to handle the increasing energy demands. When hypoglycemia is prolonged, it can overwhelm the glycogen storage and reduce blood glucose (11, 12). Therefore, blood glucose levels reflect the grade and speed of fatigue development. A modest workout begins with an increase in aerobic muscle activity; however, rigorous exercise triggers anaerobic metabolism, leading to a decrease in blood glucose and the accumulation of lactic acid (13). When mice were fed the *T. sinensis* extract, blood glucose levels were maintained at twice the level of the control group. Thus, the extract appeared to facilitate the release of glucose from glycogen and increase LDH activity, allowing the animals to regain energy after a workout.

Similar results were obtained when extracts of a fungus (4) or fenugreek (14) were used to improve exercise performance and decrease physical fatigue. Aging (15) and overloading muscle for extended periods (16) decrease LDH enzyme activity. Even though the mechanism is not clear, the *T. sinensis* extract might also prevent the decreased muscle functional capacity that accompanies aging or a workout by enhancing the enzyme activity.

Blood urea nitrogen is another blood biochemical index associated with physical fatigue. It is formed in the liver as the end product of protein and amino acid metabolism. The urea level reflects kidney function, although many other factors affect its level, including protein breakdown, dehydration, stress, and fatigue (17). After prolonged physical activity, blood urea levels normally increase. Upon giving mice the *T. sinensis* extract, blood urea levels remained as low as 73% compared to the control group. This suggests that the turtle extract prevents protein catabolism, even during intense exercise, which reflects enhanced endurance. Exhaustive heavy exercise generates free oxygen radicals (2).

The activity of GPx, a major antioxidant enzyme, was examined to determine the anti-fatigue effect of the *T. sinensis* extract. The higher enzyme activity in the extract-fed mice explains that the extract decreases oxidative stress by counteracting possible harmful aspects of radicals associated with oxidative

stress and exhaustive workouts, similar to phenolic compounds (18) and some dietary antioxidants (19). The *T. sinensis* extract enhanced the exercise capacity of mice, perhaps in part, by increasing fat utilization via decreasing triglyceride levels (to 62%) in blood. Although the mechanisms by which the extract decreased triglyceride and cholesterol levels are unclear, the effect might be beneficial during extended exercise, because better utilization of triglyceride might spare glycogen and glucose (20, 21). As a result, the extract prevented the onset of fatigue.

In combination with tonic properties of the *T. sinensis* extract, it also reduced the levels of triglyceride and total cholesterol in blood by almost half. Therefore, further studies are needed to elucidate the precise mechanisms of the *T. sinensis* extract on exercise, fatigue prevention, and reducing triglyceride and cholesterol, with the goal of developing commercial food products to manage these activities.

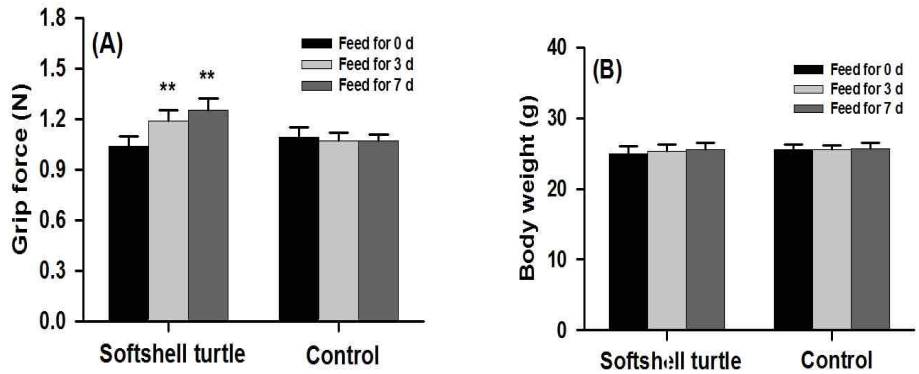

Figure 5. Effects of the softshell turtle extract on forelimb grip strength (A) and body weight (B) in mice. Values are the means±SD(n=12). **P < 0.01 compared to day 0.

Table 2. Effects of the softshell turtle (*T. sinensis*) extract on serum glucose and urea levels, LDH and GPx activities, triglyceride, and total cholesterol after the forelimb grip exercise.

	Glucose	Urea	LDH	GPx	Triglyceride	Cholesterol
Control[a]	4.9 ± 1.5	4.8 ± 0.9	725.1 ± 326.3	312.7 ± 44.4	1.3 ± 0.4	3.9 ± 1.3
Softshell turtle[a]	9.9 ± 1.0**	3.5 ± 0.8**	2,275.4 ± 697.3**	514.7 ± 153.8**	0.8 ± 0.3*	1.9 ± 0.4**
Relative	202	73	314	165	62	49

activity

(%)[b]

Glucose (mmol/L); Urea (mmol/L); Lactate dehydrogenase, LDH (U/L); Glutathione peroxidase, GPx (mU/mL); Triglyceride (mmol/L); Cholesterol (mmol/L). Relative activities are expressed as percentages of the values against the control group. Values are the means ± SD (= 12). *P<0.05 and **P<0.01 compared to control.

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Abstract (Korean)

향어*(Cyprinus carpio nudus)* 와 민물 자라*(Trionyx sinensis)*의 열수 추출물은 전통적으로 자양 강장 또는 육체적인 피로를 풀기 위해 섭취되어왔다. 이 연구에서는 두 가지의 추출물을 투여한 쥐의 운동 능력 상승효과를 확인하였다. 수영 지구력 실험과 앞발 악력 테스트는 추출물 투여량(0.5 mg/10 uL/g 몸무게)에 따라 추출물 투여 중에 수행하였으며 7일간 추출물을 투여한 결과 향어 추출물을 섭취한 쥐의 수영 지구력은 투여 0일째에 비해 52%의 유의적인 증가율[105±18 s ($P < 0.05$)]을 보였고 자라 추출물 또한 투여 0일째와 비교하여 16.8%의 근력 증가율[1.25±0.07 N ($P < 0.01$)] 을 보였다. 또한 각 추출물은 근육 증가에는 효과가 있음에도 몸무게의 변화는 거의 주지 않음을 보였다. 향어 추출물의 투여와 운동능력 실험을 수행한 쥐의 불균등화 효소인 포도당, 글루타티온 과산화효소, 초 과산화 그룹의 수치는 식염수를 투여한 쥐(control group)에 비해 각각 145%, 131%, 106%으로 유의적으로 높았다. 비만 지표인 고비중지단백-콜레스테롤 또한 128%상승하였다. 민물자라 추출물을 투여한 쥐에서는 control 그룹에 비해 혈중 포도당 수치는 202% 높음을, 요소는 73% 낮음을 보였다. 젖산 탈수효소는 314%, 글루타티온 과산화효소는 165퍼센트 증가하였다. 여기에 더해,

중성지방과 콜레스테롤은 각각 62%와 49% 증가함을 보였다. 이 연구결과는 향어 또는 민물 자라의 추출물을 섭취 하였을 시 육체적인 운동 능력과 격한 운동에 따른 산화적 스트레스를 줄여주는 효과가 있음을 알 수 있었다.