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Allium hookeri Enhances Muscular Endurance of Mice by Increasing Muscle Cross-Sectional Area

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Abstract: Despite the known physiological effects of *Allium hookeri* Thwaites (*A. hookeri*) on metabolism, the effects of ingestion of the extract on hematological parameters and exercise capacity are largely unknown. We aimed to investigate the hypothesis that water extracts of *A. hookeri* root (WEAH) would improve hematological parameters and promote exercise capacity. The intake of WEAH-containing feed significantly reduced the weight of white adipose tissue and the amount of total cholesterol in the blood, but it did not affect the body weight of mice. WEAH intake enhanced the muscular endurance of mice in treadmill endurance tests. The cross-sectional areas of muscle fibers in the gastrocnemius muscle of the WEAH-fed mice were larger than those in the control mice. WEAH promoted myoblast myogenesis by increasing the expression of the myogenic protein myosin heavy chain 3 and myotube formation. Taken together, our results suggest that *A. hookeri* may be valuable in the development of preventive and therapeutic medicines for sarcopenia as well as in providing basic knowledge of muscular functions.

Keywords: *Allium hookeri*; muscular endurance; muscle cross-sectional area; myosin heavy chain 3; myoblast.
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1. Introduction

Chronic diseases typically worsen with aging, and the most phenotypically noticeable changes affect body composition, primarily skeletal muscle, fat, and bone tissue [1]. Several studies have shown that muscle mass decreases by approximately 6% every 10 years after middle age [2]. Sarcopenia is primarily associated with visceral obesity, potentially leading to complex interactions between risk factors [3-5]. Sarcopenia reduces physical activity, which, in turn, reduces energy expenditure and increases the risk of obesity. In contrast, increased visceral fat leads to inflammation and sarcopenia. Sarcopenic obesity may have a greater effect on metabolic diseases, cardiovascular morbidity, and mortality than sarcopenia or obesity [6-9]. Therefore, a successful approach to reduce body fat while preserving and enhancing muscle mass is important for healthy aging.

Muscle formation occurs during development and when muscle tissue regenerates after damage from exercise or disease [10]. During muscle regeneration, myoblasts leave a stationary or quiescent state and differentiate through cell fusion to form muscle fibers. The expression of myogenic proteins and the length and thickness of myotubes are important factors for assessing myogenesis [11, 12]. Various myogenic proteins, such as paired box 7 (Pax7), myogenin (MyoG), and myosin heavy

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chain 3 (Myh3), indicate various stages of myogenesis (i.e., early, mid, and late, respectively) [13, 14]. Inhibition of myogenesis inhibits myotube formation, resulting in muscle loss and ultimately contributing to the development of sarcopenia [15-20]. Currently, there are no pharmacological preparations available for the treatment of sarcopenia. Discovering physiologically active substances with minimal side effects is crucial for preventing the onset of sarcopenia and supporting healthy aging.

Allium hookeri Thwaites (*A. hookeri*), a plant belonging to the Amaryllidaceae family, is rich in nutrients, especially sulfur-containing compounds such as allicin, *S*-allylcysteine, and cycloalliin [21]. *A. hookeri* has bioactive properties such as antioxidant, antibacterial, antidiabetic, anti-obesity, and hepatoprotective effects [22-26]. Moreover, our previous study showed that *A. hookeri* affects the regulation of thermogenesis [27]. However, the effects of *A. hookeri* on muscle formation and exercise capacity remain largely unknown. In this study, we investigated the effects of the water extracts of *A. hookeri* root (WEAH) on muscular endurance and muscle formation.

2. Materials and Methods

2.1. A. hookeri Root Extract Preparation

A. hookeri was purchased from Cheongsong, Gyeongsangbuk-do, Korea. SIB Identifier and herabrium material number of *A. hookeri* are 175494/1 and G, respectively. *A. hookeri* roots were washed with water, dried, and extracted by heating at 100°C for 2 h in a reflux extractor containing 1 L of primary distilled water. Subsequently, the extract was filtered under reduced pressure via a 5-μm filter paper to obtain a concentrated solution using a rotary vacuum evaporator. The powder obtained by freeze drying the concentrated solution was used as sample.

2.2. High-Resolution Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry (HR-LC-ESI-MS/MS) Analysis

WEAH analysis was performed using a C18 column on a HPLC system (Waters Q-TOF LC-MS/MS, Agilent). The mobile phase comprised 100% water+0.1% Formaldehyde (A) and 100% acetonitrile+0.1% Formaldehyde (B) at a flow rate of 0.5 mL/min. 70-min gradient elution program was employed with the following conditions: 0–40 min, 100% A and 0% B; 40–48 min, 80% A and 20% B; 48–70 min; 0% A and 100% B. The injection volume was 10 μL. The column oven temperature was maintained at 30°C. Data were acquired and analyzed using peak view 2.2 software (SCIEX).

2.3. Animals and Experimental Diets

Mouse husbandry conditions builds upon the methods outlined in our previous [36]. Five-week-old male C57BL mice (Dae Han Bio Link, Chungju, Korea) were divided into three groups (n=8 mice/group) as follows: standard chow diet (control), WEAH diet containing 2% of the total feed (100%), and WEAH diet containing 10% of the total feed. During the 8-week feeding period, body weight was recorded every 7 days, and food intake was recorded every 3 days.

2.4. Serum Biochemical Analysis

The effects of the experimental diets on serum aspartate aminotransferase (AST), blood urea nitrogen (BUN), glucose, total cholesterol (T-Chol), high-density lipoprotein (HDL), low-density lipoprotein (LDL), and triglyceride (TG) levels were measured using an autoanalyzer (TBA-40FR; Toshiba, Tokyo, Japan).

2.5. Treadmill Endurance Test

The treadmill endurance test was performed at a rate of 10 cm/s for 3 min with 0% slope. The speed was gradually increased to 60 cm/s and maintained thereafter. The test was performed until the mouse could not run for >10 s.

2.6. Poly-L-Ornithine and Collagen Coating

To coat the myoblast culture dishes or plates with poly-L-ornithine (PLO; Sigma-Aldrich, St. Louis, MO, USA) and collagen (Santa Cruz Biotechnology, Dallas, TX, USA), the dishes and plates were incubated 6 h at 25℃ with 0.001% solution of PLO (Sigma-Aldrich) in sterile water at 5 μg/cm2 or 0.3 mg/mL solution of collagen (Santa Cruz Biotechnology) in sterile water at 5 μg/cm2. The extra solution was aspirated, and dishes or plates were dried under UV lamp exposure and washed twice with phosphate-buffered saline (PBS).

2.7. Isolation of Mouse Primary Myoblasts

Primary mouse myoblasts were isolated from hind limb skeletal muscles of 3-day-old mouse. Muscles were separated from the bones and fat and treated with 1.5 U/mL collagenase D and 2.4 U/mL dispase solution (1:1) for 1 h to isolate myoblasts. Isolated myoblasts were centrifuged at 300×g for 5 min and resuspended in growth medium consisting of Ham's F-10 medium (WELGENE, Gyeongsangbuk-do, Korea) with 10% calf serum (WELGENE), 50 U/mL penicillin/50 μg/mL streptomycin (WELGENE), and 2.5 μg/mL fibroblast growth factor (Peprotech, Rocky Hill, NJ, USA). Primary mouse myoblasts were cultured in PLO (Sigma-Aldrich)-coated dish for culture or collagen (Santa Cruz Biotechnology)-coated dish to induce myogenesis at 37℃ in a humidified 5% CO2 atmosphere. When the cells reached 100% confluence, the culture medium was replaced with Dulbecco's modified Eagle's medium (WELGENE) supplemented with 2% horse serum (WELGENE) and 50 U/mL penicillin/50 μg/mL streptomycin (WELGENE). During induction of myogenesis, the medium was replaced with fresh medium every 2 days.

2.8. Immunoblot Analysis

Whole cells were lysed in the Mammalian Protein Extraction Reagent buffer (Thermo Fisher Scientific, Middlesex, MA, USA) with protease inhibitor cocktail (Roche Applied Science, Schlieren, Switzerland). Proteins were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and transferred to a nitrocellulose membrane (0.45 μm; Merck Millipore, Billerica, MA, USA). The membrane was blocked with 5% skimmed milk (Rockland Immunochemicals, Limerick, PA, USA) in PBS containing 0.05% Tween-20 (PBST) for 1 h and then incubated with the primary antibodies for 16 h at 4°C. After washing, the blots were incubated with horseradish peroxidase–conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA, USA) for 1 h at 20°C. The proteins were detected using the SuperSignal system (Thermo Fisher Scientific) with a chemiluminescence imaging system (Luminograph I; ATTO, Tokyo, Japan). The primary antibodies used were anti-glyceraldehyde 3-phosphate dehydrogenase (1:2000; Meridian Life Science, Memphis, TN, USA), anti-Myh3 (1:500; Santa Cruz Biotechnology), and anti-Pax7 (1:500; Santa Cruz Biotechnology). Immunoblot bands were quantified using the ImageJ software (NIH, Bethesda, MD, USA).

2.9. Immunofluorescence Microscopy

Primary mouse myoblasts were plated in a 4-well collagen-coated chamber slide, and myogenesis was induced for 5 days. Primary mouse myoblasts were fixed in 4% paraformaldehyde for 10 min and permeabilized with 0.5% (v/v) Triton X-100 in PBS for 15 min. All samples were blocked with 5% goat serum and 0.1% bovine serum albumin for 1 h, followed by incubation with the primary antibody overnight at 4°C. After washing the slides four times for 10 min each, Alexa Fluor 488–conjugated goat antibodies against mouse IgG (Thermo Fisher Scientific) were used as secondary antibodies, and the nuclei were co-stained with 4′,6-diamidino-2-phenylindole (Thermo Fisher Scientific). The slides were then washed four times with PBST for 10 min each and mounted with ProLong Gold anti-fading mounting medium (Thermo Fisher Scientific). Images were acquired using a Zeiss LSM 880 Meta confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany). The primary antibody used in this study was mouse monoclonal anti-Myh3 (1:500; Santa Cruz Biotechnology).

2.10. Statistical Analysis

Data are presented as mean±standard deviation. Statistical analyses were performed using two-tailed Student's t-tests. Differences were considered statistically significant at $p<0.05$.

3. Results

3.1. HR-LC-ESI-MS/MS Identification for Compounds in WEAH

HR-LC-ESI-MS/MS analyses were performed to determine the composition of WEAH (Figure 1). The results of the HR-LC-ESI-MS/MS analysis showed that the composition of WEAH was confirmed to consist of arginine, methiin, cycloalliin, alliin, isoalliin, adenine, tyrosine, adenosine, deoxyadenosine, guanosine, guanine, and tryptophan (Table 1).

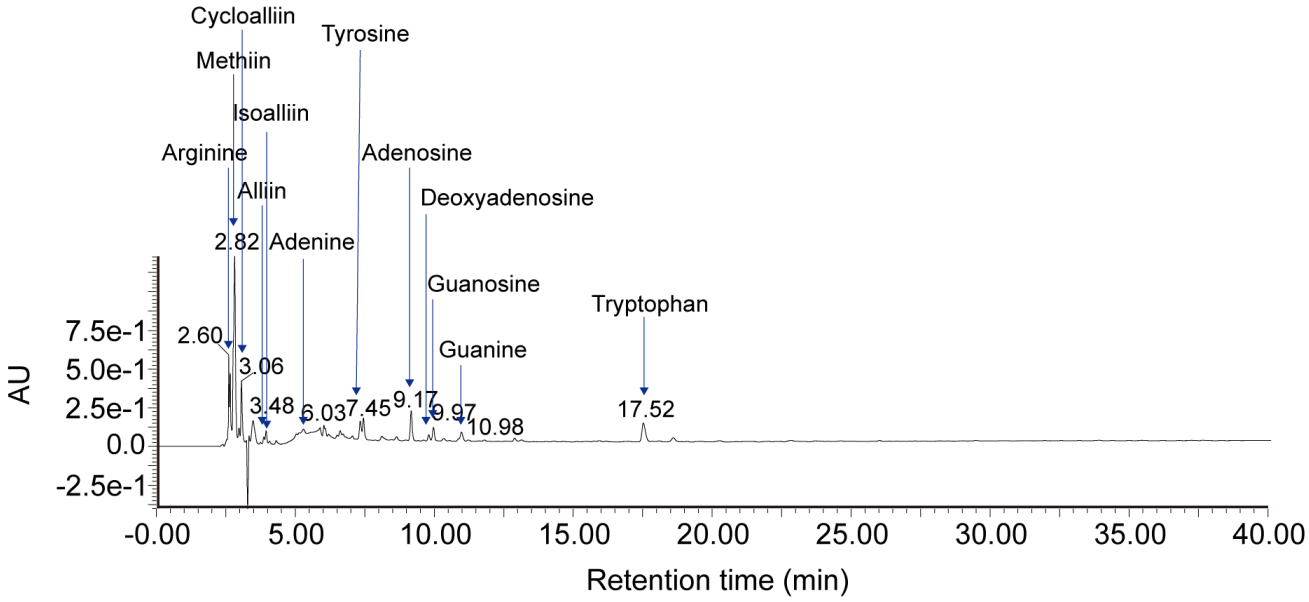

Figure 1. Chromatogram of water extracts of *Allium hookeri* root

Table 1. Compounds identified in the HR-LC-ESI-MS/MS profile of the water extracts of *Allium hookeri* root

No.	Retention time (min)	Analyte	Molecular formula	Molecular weight	Mass ion	Precursor ion (*m/z*)
1	2.6	Arginine	$C_6H_{14}N_4O_2$	174.2010	$[M+H]^+$	175.1195
2	2.8	Methiin	$C_4H_9NO_3S$	151.1842	$[M+H]^+$	152.0376
3	3.1	Cycloalliin	$C_6H_{11}NO_3S$	177.2214	$[M+H]^+$	178.0530
4	3.9	Alliin	$C_6H_{11}NO_3S$	177.2214	$[M+H]^+$	178.0530
5	4.0	Isoalliin	$C_6H_{11}NO_3S$	177.2214	$[M+H]^+$	178.0530
6	5.2	Adenine	$C_5H_5N_5$	135.1267	$[M+H]^+$	136.0619
7	7.3	Tyrosine	$C_9H_{11}NO_3$	181.1885	$[M+H]^+$	182.0829
8	9.2	Adenosine	$C_{10}H_{13}N_5O_4$	267.2413	$[M+H]^+$	268.1047
9	9.8	Deoxyadenosine	$C_{10}H_{13}N_5O_3$	251.2419	$[M+H]^+$	252.1088
10	10.0	Guanosine	$C_{10}H_{13}N_5O_5$	283.2407	$[M+H]^+$	284.1018
11	10.9	Guanine	$C_5H_5N_5O$	151.1261	$[M+H]^+$	152.0572
12	17.5	Tryptophan	$C_{11}H_{12}N_2O_2$	204.2252	$[M+H]^+$	205.0968

3.2. WEAH Reduces Visceral Fat in Mice

Mice were fed WEAH containing 2% or 10% of their total diet for 8 weeks, followed by physiological analysis to determine the effect of WEAH on metabolic activity. Food intake and body, spleen, and gastrocnemius weights did not differ between the groups (Figure 2A–D), but white adipose tissue (WAT) significantly decreased in the WEAH 2% and 10% groups compared with the control group (Figure 2E). In addition, the AST, BUN, glucose, TG, HDL, and LDL levels determined through serum analysis were similar among the groups (Figure 3A–F). Conversely, T-Chol levels were significantly lower in the WEML 10% group than in the control group (Figure 3G). These results suggest that WEAH enhances vascular health by reducing the WAT.

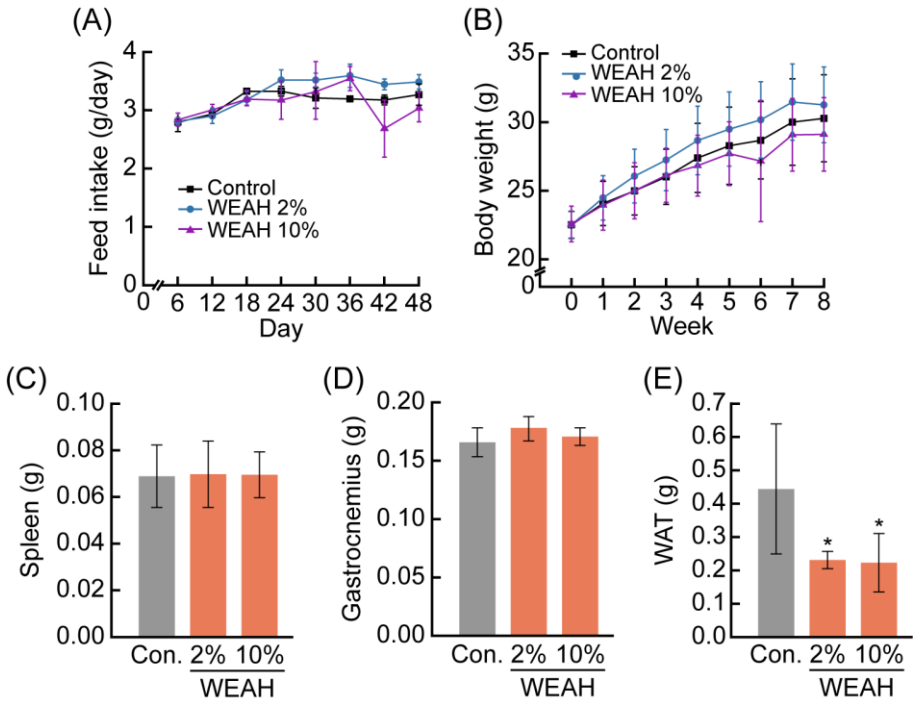

Figure 2. Effect of water extracts of *Allium hookeri* root (WEAH) on metabolic activity in mice. Mice were provided with WEAH containing 2% or 10% of total feed for 8 weeks. Changes in food intake (A), body weight (B), spleen weight (C), gastrocnemius weight (D), and white adipose tissue (WAT) weight (E) were measured. All experimental data are presented as mean±standard deviation (n=8/group). *p<0.05.

3.3. WEAH Enhances Muscle Endurance by Increasing the Muscle Cross-Sectional Area in Mice

To analyze the muscular endurance function of the WEAH-fed mice, a treadmill endurance test was performed by measuring the running distance and exhaustion time (Figure 4A and Supplementary video 1). The results of the treadmill endurance test showed that the running time before exhaustion was longer in the WEAH 2% (29.6±1.6 min) and WEAH 10% groups (32.9±1.3 min) than in the control group (23.0±2.1 min) (Figure 4B). Furthermore, the WEAH 2% (480.3±41.9 m) and WEAH 10% groups (574±51.4 m) ran longer distances before exhaustion than the control group (324.7±52.8 m) (Figure 4C). These results suggested that WEAH contributed to the improvement of muscular endurance in mice.

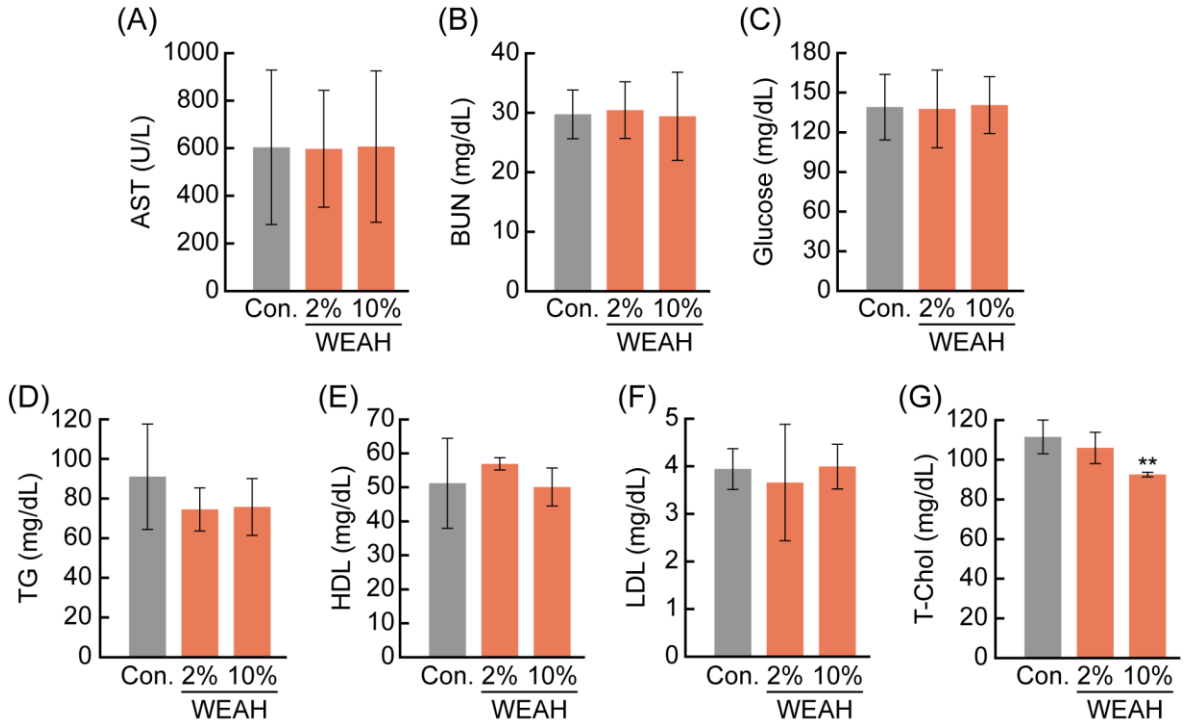

Figure 3. Effects of water extracts of *Allium hookeri* root (WEAH) on the blood profile of mice. Mice were provided with WEAH containing 2% or 10% of total feed for 8 weeks. Changes in aspartate aminotransferase (AST) (A), blood urea nitrogen (BUN) (B), glucose (C), triglyceride (TG) (D), high-density lipoprotein (HDL) (E), low-density lipoprotein (LDL) (F), and total-cholesterol (T-Chol) (G) levels were measured. All experimental data are presented as mean±standard deviation (n=8/group). **p<0.01.

Next, as muscular endurance increased in the WEAH-fed mice, muscle fiber size and muscle fiber size distribution were measured through hematoxylin and eosin staining (Figure 5A). The average muscle fiber area of the WEAH 10% group was 3512.8±1077.5 μm^2, which was larger than that of the control group (2579.0±1163.4 μm^2) (Figure 5B). Moreover, the muscle fiber size distribution in the WEAH 10% group appeared to shift toward larger fiber diameters (Figure 5C). The composition of hypertrophic muscle fibers (>5000 μm^2) was higher in the WEAH 10% group (25.6%) than in the control group (11.0%), respectively. Taken together, these results suggest that WEAH contributes to muscular endurance through histological changes in the muscles.

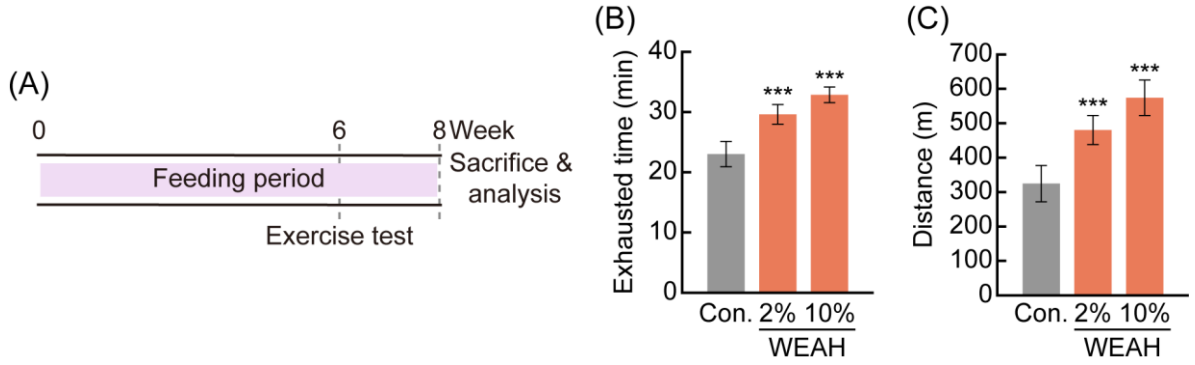

Figure 4. Effects of water extracts of *Allium hookeri* root (WEAH) on changes in exercise ability in mice. Mice were provided with WEAH containing 2% or 10% of total feed for 8 weeks. (A) Experimental scheme for experimental diet intake. (B, C) Treadmill endurance tests were performed with progressively increasing speed and time. All experimental data are presented as mean±standard deviation (n=8/group). ***p<0.001.

3.4. WEAH Promotes Myogenesis of Primary Mouse Myoblasts

To examine the myogenic efficacy of WEAH, mouse primary myoblasts were induced to differentiate in the presence of 2 mg/mL WEAH for the indicated periods, and immunoblot analysis was performed using anti-Myh3 and anti-Pax7 antibodies. Myh3 expression significantly increased 3 days after WEAH treatment, whereas Pax7 expression significantly decreased 3 days after WEAH treatment (Figure 6A). Furthermore, treatment with WEAH increased myotube formation (Figure 6B).

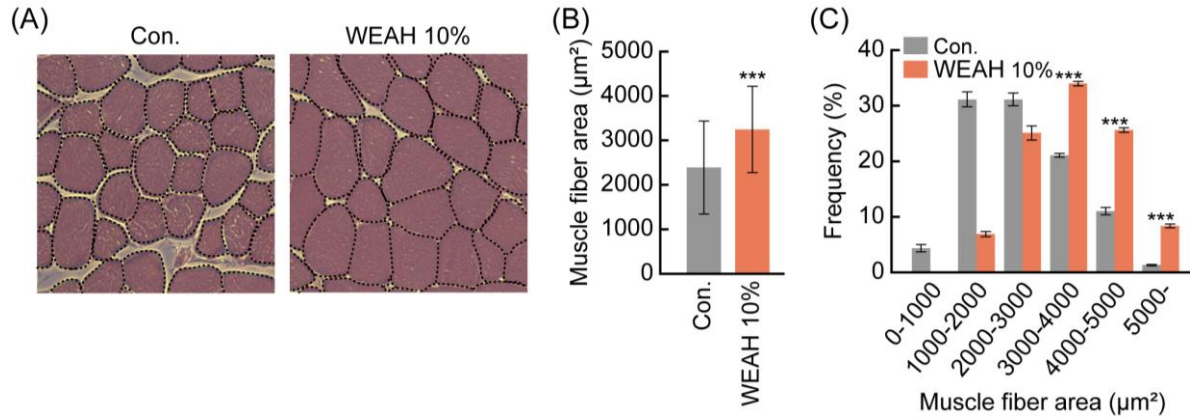

Figure 5. Effects of water extracts of *Allium hookeri* root (WEAH) on muscle fiber composition in mice. (A) Representative histological image of hematoxylin and eosin-stained cross section of the gastrocnemius muscle in experimental mice. (B, C) Cross-sectional area of the myofibers of the gastrocnemius muscle from each group. The average fiber area (B) and frequency distributions (C) of fibers were measured and compared. All experimental data are presented as mean±standard deviation (n=8/group). ***p<0.001.

4. Discussion

In this study, we showed that WEAH reduced total cholesterol levels by reducing WAT and improving blood profiles in mice. Furthermore, WEAH significantly enhanced muscular endurance and promoted muscle formation by increasing the muscle cross-sectional area. The positive physiological effects of WEAH provide fundamental evidence for its potential as a therapeutic agent against sarcopenia.

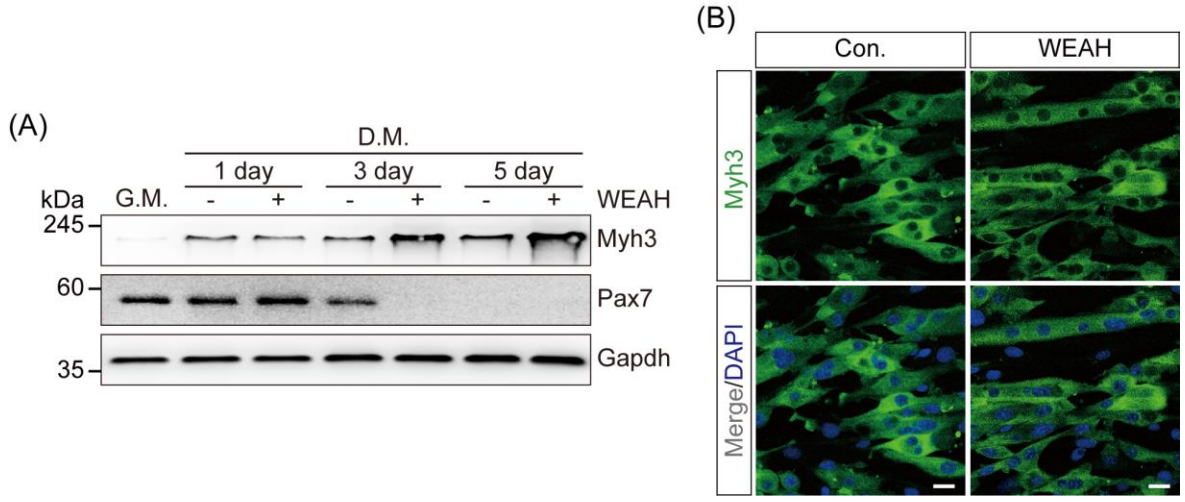

Figure 6. Effect of water extracts of *Allium hookeri* root (WEAH) on myogenesis in primary mouse myoblasts. (A) Primary mouse myoblasts were treated with 2 mg/mL of WEAH for the indicated periods in differentiation medium, and cell lysates were immunoblotted using the indicated antibodies. Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) served as a loading control. (B) Primary mouse myoblasts were treated with 2 mg/mL WEAH in the differentiation medium to induce differentiation for 5 days; then, immunofluorescence analysis was performed. Myosin heavy chain 3 (Myh3; green) and 4′,6-diamidino-2-phenylindole (DAPI; blue) were analyzed as representative markers for myotube and nuclear staining, respectively. Scale bars, 20 μm.

There is growing interest in harnessing the potential of herbal supplements to enhance muscle mass and overall health in individuals with sarcopenia [28]. A recent study highlighted various herbal compounds that can affect skeletal muscle [29]. Some herbal compounds have demonstrated modest effects on skeletal muscles in humans. Notable examples include curcumin from *Curcuma longa*, alkaloids and steroid lactones from *Withania somnifera*, catechins from *Camellia sinensis*, and gingerols and shogaols from *Zingiber officinale*. However, a comprehensive understanding of the precise mechanisms underlying the enhancement of muscular endurance and promotion of muscle formation by these natural products is lacking, and studies on natural products that can increase muscle mass and reduce visceral fat are scarce.

A. hookeri is widely consumed as a medicinal herb owing to its diverse physiological properties [30]. Studies have shown that *A. hookeri* suppresses fat accumulation in high-fat diet–induced obese mice and improves metabolic changes by regulating the expression of genes involved in cholesterol synthesis and fat metabolism [22, 26]. We showed that WAT was reduced in WEAH-fed mice. Based on these insights, we investigated the effects of WEAH on hematological parameters. WEAH-fed mice had a positive blood profile and reduced total cholesterol levels. As aging progresses, there are noticeable changes in the body composition, including bone, fat, and muscle [31, 32]. These changes are accompanied by a progressive decrease in muscle and bone mass, an increase in total body fat, an increase in visceral fat, and fatty infiltration into the skeletal muscle, bone marrow, and liver. This complex interplay contributes to elevated cholesterol levels and increases the risk of metabolic diseases such as atherosclerosis [33]. The reduction in WAT and total cholesterol observed in WEAH-fed mice indicates that WEAH has the potential to reduce visceral fat while improving blood profiles.
The consumption of WEAH significantly improved the muscular endurance of mice in the treadmill endurance test. Following the confirmation of improved muscular endurance, we analyzed the gastrocnemius muscle of the mice. Although there were no significant differences in gastrocnemius weight among the groups, cross-sectional analysis revealed a notable increase in average muscle fiber thickness in the WEAH-fed group. Given that the function of muscle tissue is greatly influenced by the thickness of each muscle fiber rather than the quantity of muscle fibers, the improvement in muscular endurance is attributed to the increase in muscle fiber thickness [34, 35]. Myogenesis is the process by which myoblasts form muscle tube cells through cell fusion, eventually forming muscle

fibers [11,12]. Changes in the expression of myogenic proteins indicate the progression of myogenesis, allowing the assessment of muscle formation based on these changes. We confirmed that Myh3 expression increased when primary mouse myoblasts were treated with WEAH. In addition to the increased expression of Myh3, the expression of Pax7 decreased, confirming that muscle formation was significantly promoted. Thiosulfinates and cysteine sulfoxides, produced by the alliinase system, are secondary metabolites found within the WEAH of the *Allium* genus [36, 37]. The primary constituents of WEAH were confirmed to consist of alliin, isoalliin, cycloalliin, and methiin which belong to cysteine sulfoxide, a secondary metabolite, through HR-LC-ESI-MS/MS analysis. Alliin is known to have a variety of bioactive properties, including anti-diabetic and anti-inflammatory properties. Similarly, isoalliin, an isomer of alliin, exhibits similar properties [38-42]. In addition, cycloalliin and methiin are known to have physiological activities such as antihyperlipidemia. [43]. However, further investigation of the various active ingredients present in WEAH is required, and the ingredients that reduce WAT and improve muscle endurance should be also identified. In conclusion, our findings showed that WEAH can improve muscle endurance while reducing visceral fat, thereby raising expectations as a substance that improves overall human health, including sarcopenic obesity.

Acknowledgments

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Author contributions

Conceived and designed the experiments: K.K.K.; Performed the experiments: D.J., S.L.; Analyzed the data: D.J., K.K.K.; Contributed materials: J.P.K.; Wrote the paper: D.J., K.K.K.; All authors read and approved the final manuscript.

Ethical statement

Research and animal care protocols were approved by the Animal Experimental Ethics Committee of the Chungnam National University (Daejeon, Korea, approval no. 202304A-CNU-058) and were performed in accordance with the institutional guidelines.

Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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