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RESEARCH PAPER

AHCC Supplementation Attenuates Muscle Atrophy via Akt Activation in Hindlimb-suspended Rat

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Abstract We investigated the inhibitory effect of active hexose correlated compound (AHCC) on muscle atrophy in hindlimb-suspended rats. Twenty-four six-week-old male Sprague-Dawley rats were randomly divided into three groups: the control sedentary group (CS, n = 8), the hindlimb-suspended group (HS, n = 8), and the hindlimb-suspended and AHCC-supplemented group (HSA, n = 8). Hindlimb suspension and AHCC supplementation were performed for two weeks. The HSA group was treated with AHCC (1 g/1 kg of body weight (BW)) orally in 0.3 mL of PBS solution, while the HS group received the vehicle (PBS solution) only. After two weeks, the cross-sectional area (CSA) of the HS and HSA groups decreased by approximately 36% ($p < 0.05$) and 19%, respectively, compared to the CS group. In addition, myonuclear numbers of the HS and HSA groups and the extensor digitorum longus (EDL) muscle weight of the HS group decreased 30% ($p < 0.05$) and 18%, respectively, compared to the CS group. AHCC supplementation increased the phosphorylation of pAkt/Akt in the HSA group compared to the HS group ($p < 0.05$). Furthermore, Fbx32 and MuRF1 protein expression in the HSA group recovered to the level of the CS group. Based on these results, AHCC supplementation may have a positive role in the prevention of muscle atrophy via Akt activation in hindlimb-suspended rats.

Keywords: AHCC, hindlimb suspension, myonuclei, CSA, muscle atrophy

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1. Introduction

Muscle atrophy is defined as the loss of muscle mass and strength and a decline in physical performance due to various causes. Bed rest, immobilization, denervation, and aging are known to induce skeletal muscle atrophy and result in restricted locomotion, muscle weakness, osteoporosis, and increased mortality [1]. In addition, muscle atrophy is closely related to various metabolic syndromes, including obesity [2], hypertension [3], dyslipidemia, [4] and insulin resistance [5].

Previous studies demonstrated that skeletal muscle atrophy was induced by protein degradation via ubiquitination pathways involving a variety of atrogenes and decreased protein synthesis [6]. It is well known that Akt plays a crucial role in protein synthesis by activating the IGF1–Akt–mTOR pathway and induces growth, proliferation, and cell survival in skeletal muscle [7]. pAkt suppresses FoxO transcriptional factors, which are expressed upstream of Fbx32 and MuRF1 associated with muscle atrophy factors [8]. In previous studies, increased Fbx32 and

MuRF1 protein expression was found in skeletal muscles under conditions of fasting, denervation, hindlimb unloading, and dexamethasone treatment [9,10].

Conversely, several reports have suggested that atrogenes were decreased because of decreased myonuclear numbers in muscle atrophy [11-15]. During muscle atrophy, the muscle cross-sectional area (CSA) and myonuclei were progressively reduced. In addition, reduced myonuclear numbers decreased the transcriptional activity and protein synthesis in muscle fiber [16,17]. However, exercise and supplementation with amino acids and proteins facilitated the maintenance of myonuclei and attenuation of muscle atrophy [18,19].

Active hexose correlated compound (AHCC) is an extract of *Lentinula edodes* of the Basidiomycetes family of fungi. It is a mixture of amino acids, minerals, and polysaccharides. AHCC is mainly composed of acetylated α-glucan, which is relatively easier to absorb than β-glucan. The main components of AHCC are oligosaccharides, which are enriched in low molecular weight acetylated a-1,4-glucans. The bioactive effects of AHCC have been attributed to its glucan fraction [20]. AHCC supplementation was not associated with any side effects in humans [21,22]. It is composed of 74% oligosaccharides and 20% alpha (-1, 4-) linked glucans with an acetylated hydroxyl group, underlying the biological activity or immune response [22].

Thus, AHCC has been shown to exhibit potential anti-cancer effects in animal and human studies [23-26]. With regard to myopathy, AHCC supplementation prevented disease progression in Leishmania infantum-infected dogs characterized by myopathies involving muscle degeneration [27]. Also, AHCC supplementation decreased mortality and increased the time to death and increased the ability to clear bacteria in hindlimb unloading and mice infected with *Klebsiella pneumonia* [28].

However, the effects of AHCC supplementation on skeletal muscle atrophy have yet to be reported. Therefore, in the present study, we investigated the effect of AHCC supplementation on histological changes and muscle atrophy-related protein expression in hindlimb-suspended rats.

2. Material and Methods

2.1. Experimental animals and diets

All experimental protocols were approved by the animal study committee of Sunmoon University (SM- 2011-2-3). Twenty-four male Sprague-Dawley rats were purchased from Samtaco Bio Korea (Hwaseong, Korea). Following a one-week acclimation period, the rats were used at 6-weeks of age. The rats were randomly divided into three groups: the CS, control sedentary group (n = 8); the HS, hindlimb-suspended group (n = 8); and the HSA, hindlimb-suspended plus AHCC-supplemented group (n = 8). The rats were housed in individual cages at controlled temperature (23 ± 1°C) and humidity (50 ± 5%) with a 12-h light-dark cycle. They were provided free access to tap water and food. Their diet (Purina, Korea) was composed of 20% protein, 5% fat, 60% carbohydrates, 5% fiber, 3.5% minerals, and 1.0% vitamin mix based on the AIN-76G diet.

2.2. AHCC supplementation

AHCC was kindly provided by Amino Up Chemical in Japan. The HSA group was treated with AHCC (1 g/1 kg body weight (BW) per day) orally in 0.3 mL of PBS solution, while the HS group received the vehicle (PBS solution) only for two weeks.

2.3. Hindlimb suspension

Hindlimb suspension was performed according to a standard rodent hindlimb unloading model as described in a previous study [29]. Briefly, the animals were maintained in individual cages with their tail attached to the top of the cage using tape to achieve 30° suspension of the hindlimbs between the floor and the body of the animal. The suspended animals were free to walk using their front limbs and were allowed access to food and water ad libitum. Any abnormalities of the rats, including tails, eyes, and facial appearance, were evaluated daily to detect stress. The experiment was interrupted following any indication of pain or discomfort in the animals. At the end of the two-week experimental period, the rats were anesthetized with diethyl ether after fasting for 12 h. Tissue samples were stored at -80°C until further analysis.

2.4. Histological analysis

Extensor digitorum longus (EDL) muscle samples were fixed in 4% paraformaldehyde, followed by paraffin embedding. Muscle samples (10-μm thickness) were cut from the paraffin blocks using a manual microtome (Leica RM2125RTS, Milan, Italy). The slides obtained from sections of the central portion of the EDL muscle were stained with hematoxylin and eosin (HE). Photographs were obtained using an optical microscope (Olympus, CKX 41, Munster, Germany) attached to a digital camera (Canon, A640). The images were analyzed (100 fibers per muscle) using the image morphometry program (ImageJ, NIH, Bethesda, MD, USA). The CSA of and myonuclear numbers in the EDL muscle fibers were calculated according to the method of Bodine and Baar [30].

2.5. Western blots

The total EDL muscle protein was extracted and prepared

according to Baghirova *et al*. [31]. The protein concentration was determined by the Bradford Protein Assay (Bio-rad, Hercules, CA, USA). Briefly, proteins were transferred to polyvinylidene difluoride membranes (PVDF) (Millipore, Bedford, MA, USA) and blocked for 1 h at room temperature in 5% BSA using Tris-buffered saline in Tween-20 (TBST). Primary antibodies used included: pAkt^{s473} (Cell Signaling, Beverly, MA), Akt (Cell Signaling, Beverly, MA), MuRF1 (Abcam, Cambridge, MA), or Fbx32 (Abcam, Cambridge, MA). Primary antibody reactions were performed for 2 h at room temperature in 5% BSA, followed by incubation with a secondary antibody of either HRP-conjugated anti-goat IgG or anti-rabbit IgG (Santa Cruz, CA, USA) for 1 h. The target proteins were identified or detected using an ECL kit (GE Healthcare, Buckinghamshire, UK). The pAkt ^{s473} and Akt antibodies were analyzed by calculating the ratio between the phosphorylated proteins and the total protein (pAkt/Akt). The obtained films were then scanned (Pixma E510 scanner, Canon Inc, Japan) and densitometric analysis of the bands was performed with the Quantity One program (Bio-rad, Hercules, CA, USA).

2.6. Statistical analyses

All data were analyzed using SPSS software (version 15.0 for windows). The data are expressed as the mean ± SE and the values were analyzed by one-way ANOVA, followed by the LSD (least significant difference) test. Significance was defined as $\alpha = 0.05$.

3. Results

3.1. Body and tissue weights

As shown in Table 1, the final body weights of the HS and HSA groups were significantly lower than those of the CS group ($p < .01$). In addition, the EDL muscle mass was

Table 1. Body and tissues weights

	CS	HS	HSA
Initial Body mass (g)	204.9 ± 5.71	205.2 ± 3.28	205.7 ± 3.24
Final Body mass (g)	311.3 ± 6.19	200.1 ± 9.42*	199.7 ± 12.9*
EDL (mg)	139.7 ± 0.01	89.8 ± 0.01**	92.3 ± 0.01**
Soleus (mg)	112.3 ± 0.01	84.2 ± 0.01**	93.2 ± 0.01**
PFT (g)	3.4 ± 0.52	0.5 ± 0.13**	0.4 ± 0.10**
EFT (g)	3.4 ± 0.28	1.2 ± 0.35**	1.2 ± 0.21**

$^{*}p < 0.05$, $^{**}p < 0.01$ vs CS. EDL: extensor digitorum longus, PFT: perirenal fat tissue, EFT: epididymal fat tissue.

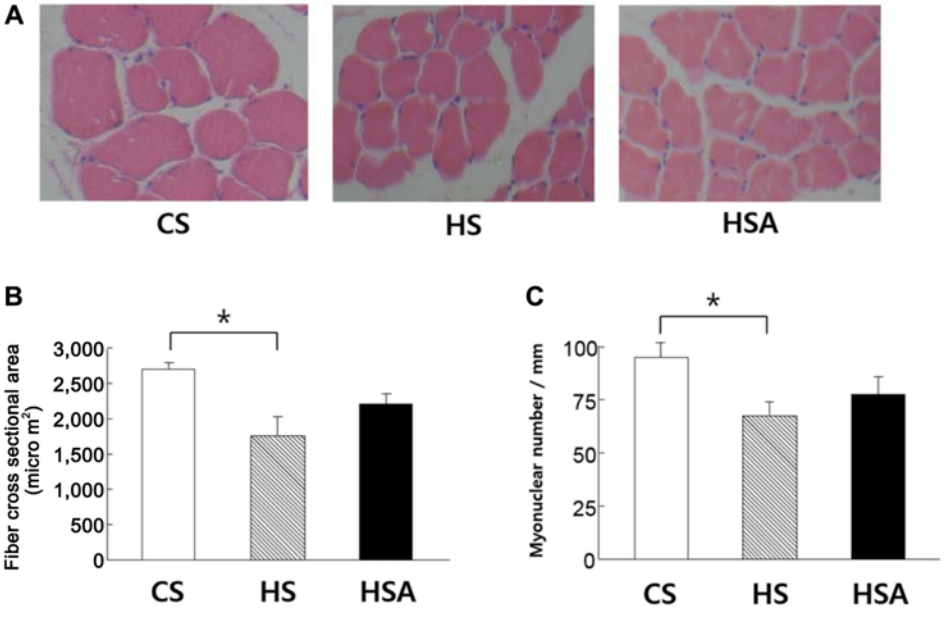

Fig. 1. Muscle fiber size. (A) Cross-sections of EDL muscle stained with H&E in the CS, HS, and HSA groups. The mean fiber cross-sectional area (μm^2) of EDL (B) and the number of myonuclei per millimeter of fiber length (C). $^{*}p < 0.05$, significantly different between the CS and HS groups. The data are presented as the mean ± SE. CS (n = 8): control sedentary group, HS (n = 8): hindlimb-suspended group, HSA (n = 8): hindlimb-suspended plus AHCC-supplemented group.

decreased by approximately 34% ($p < .01$) in the HS and HSA groups compared to the CS group. Hindlimb suspension also reduced the soleus muscle mass in all groups by 17 to 25% ($p < .01$). The peri-renal and epididymal fat weights of the HS and HSA groups were significantly different than those of the CS group ($p < .01$). However, there were no significant differences in body weight or tissue weights between the HS and HSA groups.

3.2. Effect of AHCC supplementation on CSA and myonuclear numbers

As shown in Fig. 1A and 1B, after 14 days of hindlimb suspension, the CSA of the HS group decreased significantly by approximately 64% compared to the CS group ($p < .05$). However, there were no significant differences between the HS and the HSA groups ($p = 0.098$). In addition, the CSA of the HSA group was 81% of the value in the CS group and showed no significant difference between the groups. Fig. 1C represents the myonuclear numbers in the EDL muscle of rats. The CSA value of the HS group was approximately 70% that of the CS group and was significantly different ($p < .05$). However, the value of the HSA group was 82% of the CS group and was not significantly different. Furthermore, there was no significant differences between the HS and HAS groups ($p = 0.347$).

3.3. Effect of AHCC supplementation on Akt and atrogenes-related proteins

As shown in Fig. 2A and Fig. 2B, there were no significant differences in the Akt and pAkt protein expressions between the groups. However, as shown in Fig. 2C, the ratio of pAkt/Akt in the HSA group was significantly increased compared to the HS group ($p < .0.01$). In addition, as shown in Fig. 3, the expression of Fbx32 (Fig. 3A) and MuRF1 (Fig. 3B) in the EDL muscle of the HS group was significantly decreased compared to that of the CS group ($p < .05$). However, the expression of FbxX32 and MuRF1 in the HSA group was significantly increased compared to the HS group ($p < .05$).

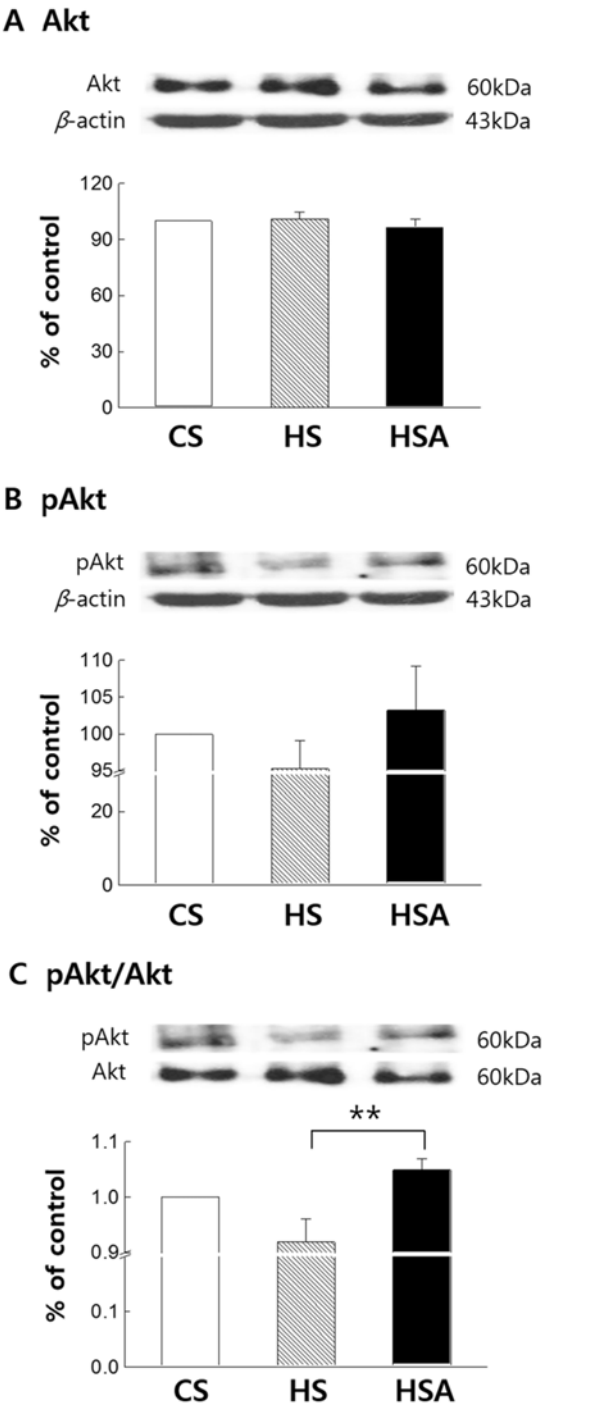

Fig. 2. Akt and pAkt protein expression and the ratio of pAkt/Akt in EDL muscle. $^{**}p < 0.01$, significantly different between the CS and HS groups. The data are presented as the mean ± SE. CS (n = 8): control sedentary group, HS (n = 8): hindlimb-suspended group, HAS (n = 8): hindlimb-suspended plus AHCC-supplemented group.

4. Discussion

In the present study, we investigated the effect of AHCC supplementation on changes in CSA and myonuclear numbers, as well as muscle atrophy-related protein expression involving pAkt/Akt, Fbx32, and MuRF1 in hindlimb-suspended rats.

In a previous study, starvation decreased the intramuscular concentration of a number of amino acids differently in the EDL and soleus muscle, decreased protein synthesis more in the EDL muscle compared to the soleus muscle, and increased expression of atrogin-1 and MuRF1 in the EDL of rats. These results indicate that the fast-twitch muscle was more sensitive compared to the slow-twitch muscle in

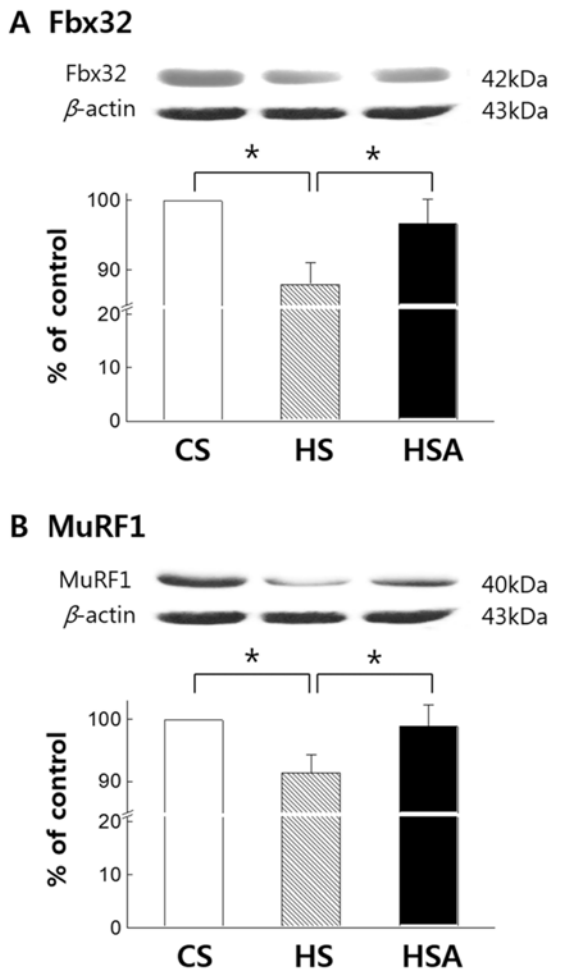

Fig. 3. Fbx32 and MuRF1 protein expression in EDL muscle. $^{*}p < 0.05$, significantly different between the CS and HS groups. The data are presented as the mean ± SE. CS (n = 8): control sedentary group, HS (n = 8): hindlimb-suspended group, HAS (n = 8): hindlimb-suspended plus AHCC-supplemented group.

muscle proteolysis [32]. Thus, we examined the effect of AHCC supplementation on the EDL muscle after hindlimb suspension in rats.

In addition, it is well known that AHCC plays a variety of physiological roles [22,26,27]. However, in the present study, we initially demonstrated the preventive role of AHCC in muscle atrophy. Our results showed that AHCC supplementation inhibited the decrease in CSA following hindlimb suspension, although the change was not significantly different. In addition, the myonuclear numbers were higher in the AHCC-supplemented group. These results suggest that AHCC plays a possible inhibitory role in muscle atrophy in hindlimb-suspended rats.

In addition to CSA and myonuclear numbers, the ratio of pAkt/Akt was increased in hindlimb-suspended rats treated with AHCC. Activation of Akt induced hypertrophy via the Akt-mTOR signaling cascade [33]. It inhibited FoxO translocation from the cytoplasm to the nucleus and reduced the transcription of target genes which regulate the ubiquitin-proteasome and autophagy-lysosome systems [34]. The pathways of Akt related to muscle hypertrophy include mTOR and glycogen synthase kinase 3β (GSK3β). Akt activation subsequently phosphorylates and activates mTOR, which then activates p70S6K and promotes muscle protein synthesis [8]. In contrast to the mTOR and GSK3β pathways, downstream of Akt is the FoxO transcription factor which controls protein degradation. A reduction in Akt activation is associated with activation of the FoxO/MAFbx pathway. Similar to our study, Dong *et al*. [35] reported that chromium treatment reduced skeletal muscle fiber size and enhanced whole-body glucose intolerance via Akt activation in hindlimb-suspended mice. The involvement of AHCC in the Akt/IL-1RI-dependent pathway, but not the IκB/NF-κB pathway, in rat hepatocytes has also been shown [36]. A previous study also showed that Akt activated cyclinD1, which increased myonuclear numbers [37]. Based on our results and previous studies, AHCC supplementation may have a crucial role in Akt activation in the prevention of muscle atrophy.

Our results demonstrated that protein degradation factors, including Fbx32 and MuRF1, in the HS group were significantly decreased compared to the CS group. The levels of these protein degradation factors in the HSA group were significantly increased compared to the HS group. Fbx32 and MuRF1 are known intranuclear atrogenes which regulate the E3 ubiquitin ligase atrogin-1/muscle atrophy F-box [38-40]. In addition, it is well known that Fbx32 and MuRF1 protein expression is increased during fasting, denervation, hindlimb suspension, and dexamethasone treatment [9,10]. However, in the present study, the expression of Fbx32 and MuRF1 was decreased in the hindlimb-suspended rats compared to non-suspended rats (Fig. 3A, B). These results were associated with decreased levels of CSA and myonuclear numbers in hindlimb-suspended rats, as mentioned above. Consistent with our results, Oishi et al. reported that two weeks of hindlimb unloading decreased myonuclear numbers in rats [41]. Similar to our study, previous studies demonstrated that mRNA levels of atrogenes, Fbx32 and MuRF1, were reduced due to decreases in myonuclei in skeletal muscle atrophy. In fact, nuclear FoxO1 and FoxO3a protein expressions were decreased when the number of myonuclei was reduced in atrophied muscles [14]. These results might be due to an atrophy-associated reduction in myonuclei, rather than a protective mechanism against further muscle atrophy-related proteins, such as Fbx32 and MuRF1. Our study was the first to demonstrate the effect of AHCC supplementation on muscle atrophy via histological

pathways and signaling mechanisms.

In conclusion, hindlimb suspension induced muscle atrophy by reducing Akt phosphorylation and resulted in reduced Fbx32 and MuRF1 protein expression in rats. However, AHCC supplementation restored the levels of CSA and myonuclear numbers by increasing skeletal muscle Akt phosphorylation of the rats. Therefore, AHCC supplementation played a crucial role in maintaining myonuclear numbers via Akt activation in hindlimb-suspended rats.

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