

Effects of Rhizome Extract of *Dioscorea Batatas* and Its Active Compound, Allantoin, on the Regulation of Myoblast Differentiation and Mitochondrial Biogenesis in C2C12 Myotubes

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Running title: Effects of yam extract and allantoin on muscle differentiation and biogenesis

Abstract: The present study was conducted to investigate the effects of rhizome extract of *Dioscorea batatas* (Dioscoreae Rhizoma, Chinese Yam) and its bioactive compound, allantoin, on myoblast differentiation and mitochondrial biogenesis in skeletal muscle cells. Yams were extracted in water and the extract was analyzed by HPLC. The expression of C2C12 myotubes differentiation and mitochondrial biogenesis regulators were determined by reverse transcriptase (RT)-PCR or Western blot. The glucose levels and total ATP contents were determined by glucose consumption, glucose uptake and ATP assays, respectively. Treatment with yam extract (1 mg/mL) and allantoin (0.2 and 0.5 mM) significantly increased of MyHC expression compared with non-treated myotubes. Yam extract and allantoin significantly increased the expression of mitochondrial biogenesis regulating proteins, PGC1 α , Sirt-1, NRF-1, and TFAM, as well as the phosphorylation of AMPK and ACC in C2C12 myotubes. Furthermore, yam extract and allantoin significantly increased the glucose uptake levels and the ATP contents. Finally, HPLC analysis revealed that the yam extract contained 1.53% of allantoin. Yam extract and allantoin, stimulated myoblast differentiation into myotubes and increased energy production through upregulation of mitochondrial biogenesis regulators. These findings indicate that yam extract and allantoin can help to prevent the skeletal muscle dysfunction through stimulation of energy metabolism.

Keywords: Allantoin; Chinese Yam; C2C12 cells; *Dioscorea batatas*; Dioscoreae Rhizoma; Myoblast differentiation; Mitochondrial biogenesis

Abbreviations: Myosin heavy chain, MyHC; Peroxisome proliferator-activated receptor gamma coactivator 1-alpha, PGC1 α ; Sirtuin 1, Sirt-1; Mitochondrial transcription factor A, TFAM; Nuclear respiratory factor-1, NRF-1; AMP-activated protein kinase, AMPK; Acetyl-CoA carboxylase, ACC; Reverse transcriptase-polymerase chain reaction, RT-PCR

1. Introduction

Dietary glucose is supplied by meals and glucose is stored as glycogen in the liver, kidney, and muscle to enable metabolic energy function. Skeletal muscle is largely responsible for regulating carbohydrate metabolism and achieving energy balance in normal feeding [1]. Accordingly, skeletal muscle function deficit, in particular age-related and disease-related muscle loss, is associated with many chronic diseases including sarcopenia, diabetes and obesity [2]. There has recently been increased interest in various functional foods and medicines taken regularly for common muscle conditions that operate through mechanisms that can alter the balance between protein synthesis and degradation and enhance muscle function [3]; however, such conditions are still difficult to control because causes of muscle loss are multifactorial and influenced by genetics.

The root (Dioscoreae Rhizoma, Chinese Yam) of *Dioscorea batatas* Decaisne (= *D. oposita* Thunberg) is a perennial trailing plant of the Dioscoreaceae family. The yam, which is one of the most important herbs in traditional medicine, has long been used as food and medication with various pharmaceutical functions. In herbology, the yam is neutral in nature, sweet in flavor, and mainly manifests its therapeutic actions in the spleen, lung, and kidney meridians [4,5]. Therefore, yams are utilized to cure yin deficiency in metabolic disorders such as diabetes and hyperthyroidism by tonifying and replenishing qi in meridian organs. Yam is also known to have digestive functions in the stomach and intestines, as well as immune regulatory and antiaging effects. In modern pharmacology, yam has been studied for its effects on asthma [6], cancer [7-9], diabetes [10,11], and liver damage [12], as well as its antioxidation, antiinflammation, and antiaging effects [13,14]. Yam contains various compounds such as dioscin [15], steroidal saponins [15,16], saponins, gallic acid, vanillic acid [13], allantoin [11], and protodioscin [17]. Allantoin, a diureide of glyoxylic acid, is an active and abundant component of yam [9,11,18]. In vivo studies have shown allantoin to have anti-asthmatic [18], anti-diabetic [11,19] and anti-hypertensive [20] activities, as well as memory-enhancing effects in Alzheimer's disease [21].

We recently conducted a study that provided scientific evidence of the abilities of various herbs to improve obesity, diabetes and sarcopenia based on clinical practice and found that some herbs have good effects that occur via regulation of the differentiation and

mitochondria biogenesis in skeletal muscle [22,23]. Therefore, in this study, we investigated the effects of yam water extract and its bioactive compound, allantoin, on myoblast differentiation and mitochondrial biogenesis in C2C12 myotubes.

2. Experimental Section

2.1. Materials

Allantoin and metformin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and penicillin/streptomycin solution were acquired from Corning (Corning, NY, USA). Fetal bovine serum (FBS), horse serum (HS) and penicillin/streptomycin (P/S) solution were obtained from Merck Millipore (Temecula, CA, USA). An ATP colorimetric assay kit was procured from BioVision Inc. (Milpitas, CA, USA). Anti-Sirt1, TFAM, NRF1, NRF2, AMPK, phospho-AMPK, total-AMPK, phospho-ACC, and total-ACC antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-MyHC and GLUT4 antibodies were acquired from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-PGC1 α antibody and RIPA buffer was obtained from Thermo Fisher Scientific (Waltham, MA, USA).

2.2. Preparation of yam extract

Dried rhizome of *D. batatas* was purchased from an herbal company (Kwangmyungdang, Ulsan, Korea) and identified by Professor Y.-K. Park, a medical botanist in herbology at College of Korean Medicine, Dongguk University (DUCOM). A voucher specimen was deposited at the herbarium of DUCOM (2017DR). Yam (200 g) were extracted by boiling in 2 L of water for 3 h, filtered through Whatman Grade 1 filter paper (Sigma-Aldrich, St Louis, MO, USA), concentrated under a vacuum rotary evaporator at 60°C, and then lyophilized in a freeze-dryer (IlShinBioBase Co., Yangju, Korea) at -80°C under 5 mTorr. Yam extract (yield=11.4%) was stored at 4°C, dissolved in 1x PBS, and filtered through a syringe filter (0.45 μ m, Corning, Germany) before being used in in vitro experiments.

2.3. Cell culture and drug treatments

C2C12 myoblasts, a mouse skeletal muscle line, were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and grown in DMEM supplemented with 10% (v/v) FBS and 1% P/S solution in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. At 85-95% confluence, myoblasts were induced to differentiate in DMEM with 2% HS once every day for an additional 5 days. The C2C12 myotubes were then treated with or without different concentrations of yam extract or allantoin. Metformin (2.5 mM) was used as a reference drug.

2.4. Western blot

After cells were lysed in ice-cold RIPA buffer containing Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific), lysates were centrifuged at 12000×g for 20 min at 4°C. Protein concentrations of the lysates were then quantified using the protein assay solution (BioRad, Hercules, CA, USA). Next, 50 µg of protein was separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose membrane. The membrane was then blocked with 5% skim milk for 1 hr at room temperature (RT), after which it was immunoblotted with primary antibodies against MyHC, PGC1 α , NRF1, NRF2, TFAM, AMPK (total or phosphor-forms), and ACC (total or phosphor forms), as well as β -actin as an internal control overnight at 4°C. Following immunoblotting, the membranes were washed three times with 1× tris-buffered saline (pH 7.4) containing 0.1% tween-20 (TBST) buffer, then reacted with horseradish peroxidase (HRP)-labeled anti-mouse or anti-rabbit IgG. All immunoblots were subsequently washed with 1× TBST three times, then developed using ECL™ Western blotting detection reagent (GE Healthcare, Pittsburgh, PA, USA). Finally, bands were detected using a BioRad ChemiDoc MP Imaging System and quantified by densitometry using the Image J programming software (NIH, Bethesda, MD, USA).

2.5. Reverse Transcriptase (RT)-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from cells by TRIzol reagent (GibcoBRL Life Technologies Inc., Grand Island, NY, USA) according to the manufacturer's instructions. The RNA concentration was then quantified using a Nano Drop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). Next, cDNA was generated from 1 µg

of total RNA using a Reverse Transcription System kit (Promega, Fitchburg, WI, USA), after which RT-PCR was conducted using a Blend Taq PCR kit (Toyobo, Osaka, Japan) and primers specific to the target genes (Table 1). For PCR, the samples were subjected to pre-denaturation at 94°C for 2 min, followed by 30 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 56°C–60°C, and extension for 1 min at 72°C. Finally, the bands were detected with the BioRad ChemiDoc MP imaging system and quantified by densitometry using the Image J programming software.

2.6. Immunocytochemistry

Differentiated myotubes were seeded on Thermanox plastic cover slips (Nunc™, Thermo Fisher Scientific) and differentiated using a common culture method for 5 days. Samples on cover slips were then fixed with 4% paraformaldehyde for 10 min, after which they were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) for 20 min. After washing with 1×PBS, cover slips were blocked with 5% bovine serum albumin (BSA) for 30 min at RT, then incubated with anti-MyHC antibody overnight at 4°C. Cover slips were subsequently labelled with AlexaFluor 488-conjugated goat anti-rabbit antibody for 1 hr at RT, then counterstained with DAPI for 5 min. Finally, the expression of MyHC was observed using a fluorescence microscope (Leica DM2500).

2.7. Glucose assay

Glucose consumption was determined in culture media using a Glucose Assay Kit (Sigma-Aldrich, St. Louis, Mo, USA). Briefly, cell culture supernatants were harvested and diluted with deionized water, after which 50 µL of diluted sample was mixed with an equal volume of assay buffer including o-dianisidine in a 96-well plate. The mixture was then incubated at 37°C for 30 min, at which time the reaction was stopped by adding 50 µL of H₂SO₄ and the absorbance (OD) at 540 nm was measured in a microtiter reader. The glucose consumption in each sample was calculated using a calculation formula from a standard curve.

Next, the cellular levels of glucose were measured in C2C12 myotubes using a Glucose Uptake Cell-Based Assay Kit (Cayman Chemical Co., Ann Arbor, MI, USA). Briefly,

C2C12 myotubes were treated with or without yam extract and allantoin at different concentrations in glucose-free medium containing 100 µg/mL of 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-d-glucose (NBDG) for 4 hr. After harvesting the cells, cell-based assay buffer (200 µL) was added to each well. The amount of 2-NBDG taken up by the myotubes was then measured with fluorescent filters that detected fluorescein (excitation/emission=485/650) using a Glomax multi detection system (Promega Biosystems, Sunnyvale, CA, USA)

2.8. ATP assay

Total ATP contents were determined using an ATP colorimetric assay kit (BioVision) according to the manufacturer's instructions. Briefly, C2C12 myotubes were harvested and homogenized in 100 µL ATP assay buffer, after which 50 µL of deproteinized cell lysate was mixed with 50 µL of reaction mix containing ATP probe, ATP converter, and developer in a 96-well plate. The mixture was subsequently incubated at RT for 30 min, at which time the absorbance (O.D.) at 570 nm was measured using a microtiter reader. Finally, the concentration of ATP (µM) in each sample was calculated using a calculation formula generated from a standard curve.

2.9. HPLC analysis

To identify allantoin in yam extract, high-performance liquid chromatography (HPLC) was conducted using an Agilent 1260 infinity II quaternary system equipped with a G7129A vial sampler and a WR G7115A diode array detector (Agilent, Waldbronn, Germany) and a ZORBAXNH2 column (4.6 × 150 mm, 5-micron). Chromatographic separation was performed using a gradient solvent system consisting of acetonitrile (HPLC grade, Merck, Darmstadt, Germany) (B) and water (HPLC grade, Merck) (A). The gradient program was as follows: 0 min, 25%B; 5 min, 17%B; 10 min, 17%B. The injection volume was 10 µL and column eluent was monitored at UV 200 nm while chromatography was performed at 30°C with a flowrate of 1.0 mL/min.

2.10. Statistical analysis

The data are presented as means \pm standard errors of means (SEMs) of three independent experiments. Differences between groups were identified by the Student's *t*-test using the GraphPad Prism program (ver. 5.0) and *P*-values < 0.05 were considered statistically significant.

3. Results

3.1. *Effects of yam extract and allantoin on myoblast differentiation into myotubes*

To investigate the effects of yam extract and allantoin on myoblast differentiation into myotubes, we determined the expression of MyHC mRNA and protein as differentiation markers in C2C12 myotubes using RT-PCR and western blot analysis. Treatment with yam extract ($P < 0.05$ for 1 mg/mL) and allantoin ($P < 0.001$ for 0.2 and 0.5 mM) significantly increased the expression of MyHC mRNA (Fig. 1A) and protein (Fig. 1B) in C2C12 myotubes compared with non-treated cells. We also observed MyHC expression in C2C12 myotubes using immunocytochemistry (Fig. 1C), which revealed that MyHC-positive myotubes with an elongated and widened cylinder-shape and multiple nuclei were present in greater numbers in yam extract and allantoin-treated cells than non-treated cells. These results indicate that yam extract and allantoin can induce myoblast differentiation into myotubes in skeletal muscle cells.

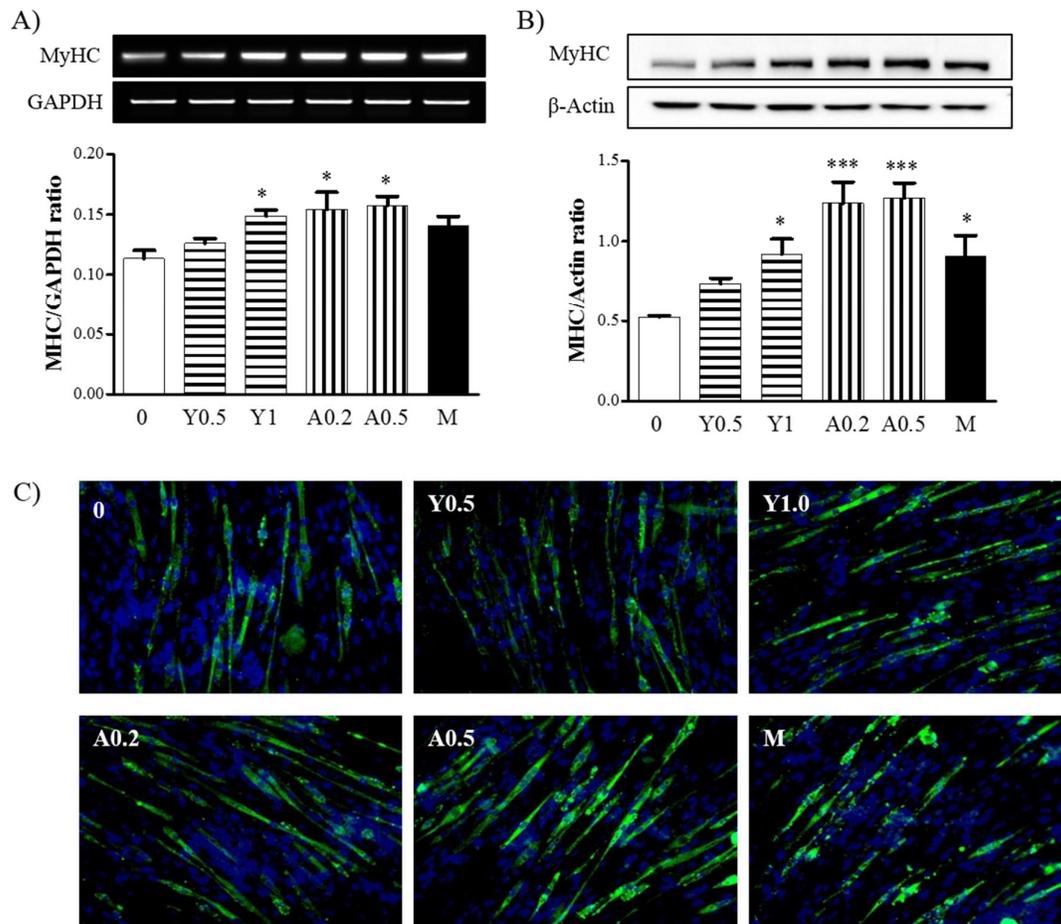


Figure 1. Effects of yam extract and allantoin on the expression of MyHC protein and mRNA in C2C12 myotubes. C2C12 myoblasts were differentiated with DMEM containing 2% horse serum for 5 days, then treated with or without yam extract (0.5 and 1.0 mg/mL) or allantoin (0.2 and 0.5 mM) for 24 hr. Metformin (2.5 mM) was used as a reference drug. The expression of MyHC mRNA (A) and protein (B) was determined by RT-PCR and Western blot, respectively. GAPDH and β -actin were used as internal controls. All data were presented as the means \pm SEM of three independent experiments. Y, yam extract; A, allantoin; and M, metformin. * p <0.05 and *** p <0.001 vs. non-treated cells. (C) The myotubes were stained with anti-MHC antibody and DAPI, then observed by fluorescence microscopy (original magnification = 200 \times). Green, MyHC-positive cells; and blue, DAPI-positive nuclei.

3.2. Effects of yam extract and allantoin on the expression of mitochondria biogenesis-regulating factors in myotubes

To investigate the effects of yam extract and allantoin on mitochondrial biogenesis in myotubes, we measured the expression of the biogenesis regulating factors, PGC1 α , NRF1, TFAM and Sirt1 mRNA and protein in C2C12 myotubes by RT-PCR and Western blot, respectively. Treatment of myotubes with yam extract (0.5 and 1 mg/mL) and allantoin (0.2 and 0.5 mM) for 24 hr increased the expression of PGC1 α (Fig. 2A,B), NRF1 (Fig. 2C,D), TFAM (Fig. 2E,F) and Sirt1 (Fig. 2G,H) mRNA (Fig. 2A,C,E,G) and protein (Fig. 2B,D,F,H). In particular, allantoin in high concentration (0.5 mM) significantly increased the expression of all regulator mRNA ($P<0.01$ for PGC1 α , $P<0.05$ for NRF-1, TFAM and Sirt1) and protein ($P<0.01$ for PGC1 α , NRF1, and TFAM, $P<0.05$ for Sirt1) compared with non-treated cells. These results indicate that yam extract and allantoin can increase mitochondrial biogenesis through upregulation of the transcription factors.

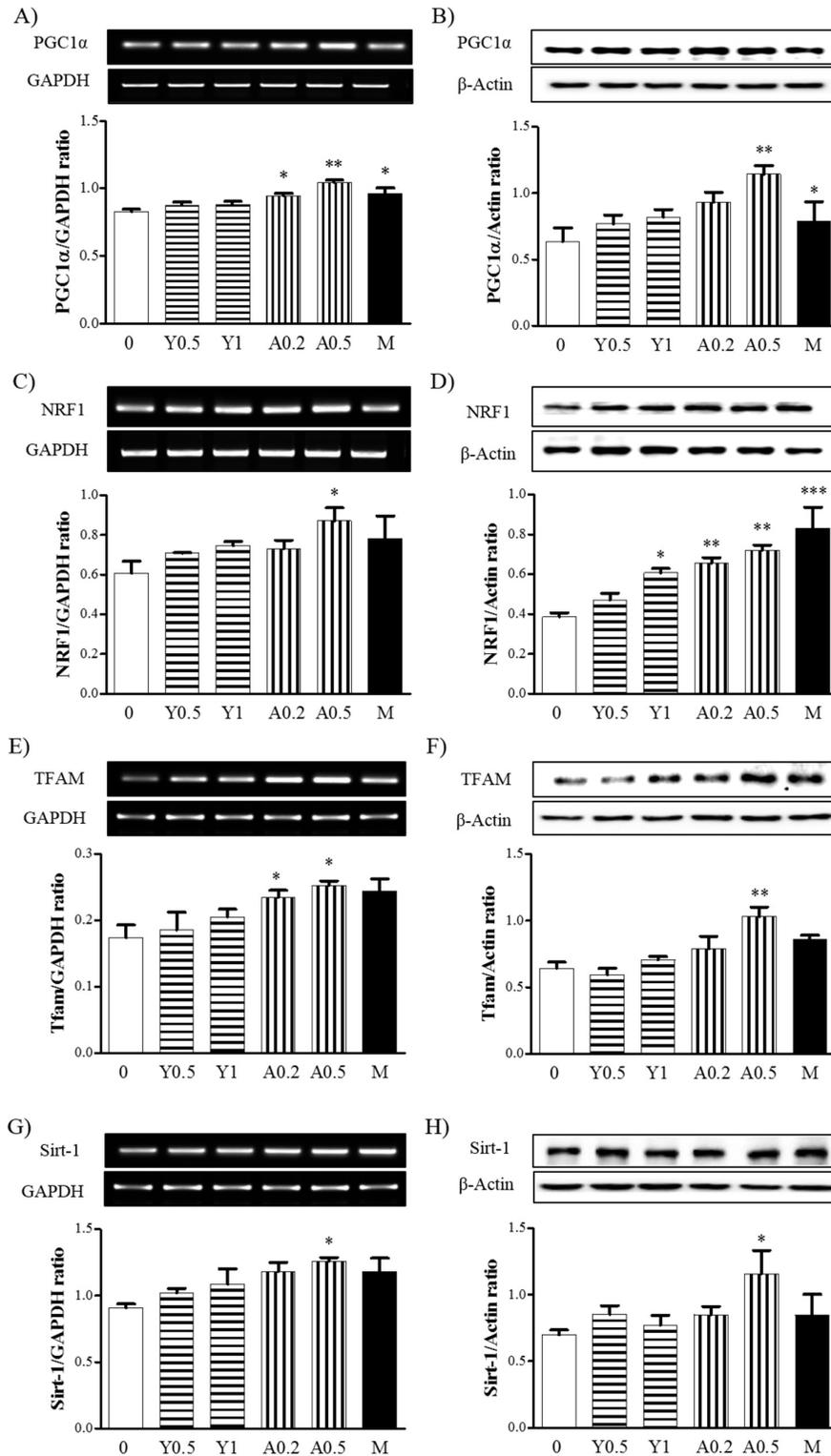


Figure 2. Effects of yam extract and allantoin on the expression of mitochondrial biogenesis-regulating factors in C2C12 myotubes. Differentiated myotubes were treated with or without

yam extract (0.5 and 1.0 mg/mL) or allantoin (0.2 and 0.5 mM) for 24 hr, after which the expression of PGC1 α (A,B), NRF-1 (C,D), TFAM (E,F) and Sirt-1 (G,H) mRNA (A,C,E,G) and protein (B,D,F,H) was analyzed by RT-PCR (A,C,E) and Western blot (B,D,F), respectively. Metformin (2.5 mM) was used as a reference drug. GAPDH and β -actin were used as internal controls. Each band was presented as a representative figure and the histogram was calculated from the band density value of each experiment. All data were presented as the means \pm SEM of three independent experiments. Y, yam extract; A, allantoin; and M, metformin. * p <0.05, ** p <0.01 and *** p <0.001 vs. non-treated cells.

3.3. Effects of yam extract and allantoin on the AMPK and ACC pathways in myotubes

Next, we investigated the effects of yam extract and allantoin on the signaling pathway activated with mitochondrial biogenesis based on evaluation of the phosphorylation of AMPK and ACC in C2C12 myotubes by Western blot. Treatment with yam extract (0.5 and 1 mg/mL) and allantoin (0.2 and 0.5 mM) resulted in increased phosphorylation of AMPK (Fig. 3A) and ACC (Fig. 3B) in the myotubes. Moreover, treatment with allantoin at high concentration (0.5 mM) significantly increased the phosphorylation of AMPK (P <0.001) and ACC (P <0.01) compared with non-treated cells. Metformin as an AMPK activator also significantly increased the phosphorylation of AMPK (P <0.001) and ACC (P <0.05) in myotubes. These results indicate that yam extract and allantoin can increase the mitochondrial biogenesis in myotubes through activation of the AMPK/ACC signaling pathway.

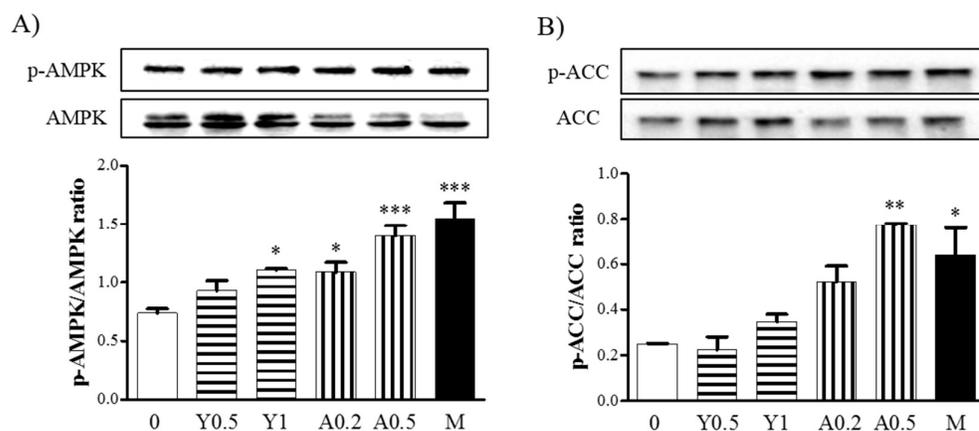


Figure 3. Effects of yam extract and allantoin on the phosphorylation of AMPK and ACC

protein in C2C12 myotubes. Differentiated C2C12 myotubes were treated with or without yam extract (0.5 and 1.0 mg/mL) or allantoin (0.2 and 0.5 mM) for 24 hr and the phosphorylation of AMPK (A) and ACC (B) protein was investigated by Western blot. Metformin (2.5 mM) was used as a reference drug. Each band was presented as a representative figure and a histogram was calculated from the band density value of each experiment. All data were presented as the means \pm SEM of three independent experiments. Y, yam extract; A, allantoin; and M, metformin. * p <0.05, ** p <0.01 and *** p <0.001 vs. non-treated cells.

3.4. Effects of yam extract and allantoin on glucose uptake in myotubes

To investigate the effects of yam extract and allantoin on glucose uptake into myotubes, we evaluated the expression of GLUT-4 protein and measured the glucose levels in culture medium and in cells using Western blot, glucose consumption assay, and glucose uptake assay, respectively. The results revealed that expression of GLUT-4 in the myotubes was significantly increased by treatment with yam extract (0.5 and 1 mg/mL) and allantoin (0.2 and 0.5 mM) in a dose-dependent manner. Moreover, allantoin treatment induced a significant increase in GLUT-4 expression (P <0.05 for 0.2 and 0.5 mM) that was more effective than yam extract treatment (Fig. 4A). Additionally, glucose was significantly decreased in culture medium of allantoin-treated myotubes (P <0.05 for 0.2 and 0.5 mM, Fig. 4B), while the cellular levels were significantly increased (P <0.05 for 0.5 mM, Fig. 4C). Metformin treatment also significantly decreased glucose levels in culture medium (P <0.01) and significantly increased cellular glucose levels (P <0.001) compared with non-treated cells. These results indicate that yam extract and allantoin can stimulate glucose uptake in myotubes by increasing the GLUT-4 expression.

3.5. Effects of yam extract and allantoin on ATP production in myotubes

To investigate the effects of yam extract and allantoin on energy production in myotubes, we measured the ATP contents in myotubes. As shown in figure 4D, treatment of myotubes with yam extract and allantoin led to dose-dependent increases in ATP production, with significantly increased ATP levels being observed in response to allantoin (P <0.05 for

0.5 mM). These results indicate that yam extract and allantoin can enhance the energy production in myotubes that might be related to upregulation of the mitochondrial biogenesis-regulating factors as shown in figure 2.

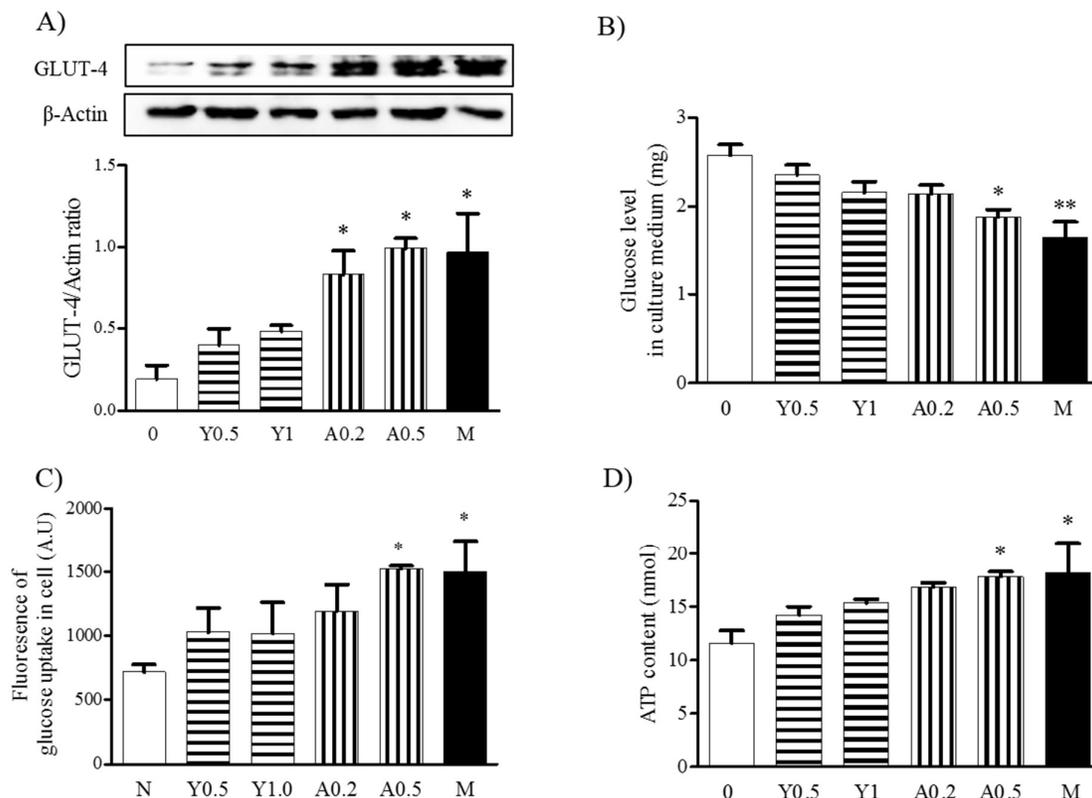


Figure 4. Effects of yam extract and allantoin on the expression of GLUT-4 and the levels of glucose in C2C12 myotubes. Differentiated myotubes were treated with or without yam extract (0.5 and 1 mg/mL) or allantoin (0.2 and 0.5 mM) for 24 hr. (A) The expression of GLUT-4 protein was determined by Western blot. Metformin (2.5 mM) was used as a reference drug and β -actin was used as an internal control. Each band was presented as a representative figure and a histogram was calculated from the band density value of each experiment. The levels of glucose in culture medium (B) and in the cells (C) were measured by a glucose consumption assay and glucose uptake assay, respectively. The contents of ATP in the myotubes were measured using an ATP assay kit (D). All data were presented as the means \pm SEM of three independent experiments. Y, yam extract; A, allantoin; and M, metformin. * p <0.05, ** p <0.01 and *** p <0.001 vs. non-treated cells.

3.6. HPLC analysis

To analyze the content of allantoin in yam extract, we conducted HPLC analysis and then compared the retention time of samples with that of authentic standard (Fig. 5A). The content of allantoin in yam water extract was subsequently calculated by comparison of peak areas (Fig. 5B). The equation of the calibration curves for allantoin was $y=16039x - 16.79$. In addition, the correlation coefficient of the calibration curve was higher than 0.9995 at concentrations of 0.0225-0.36 mg/mL and the concentration of allantoin in the extract was 1.53%. The relative standard deviation of precision and repeatability was 0.67% and 1.85%, respectively.

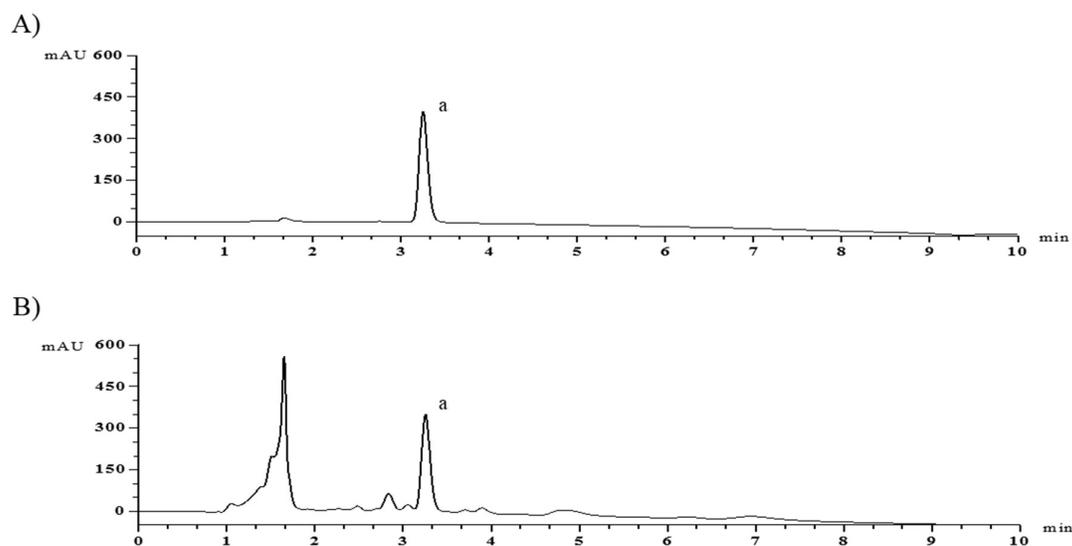


Figure 5. HPLC analysis of allantoin in the yam extract: (A) allantoin as a standard compound, and (B) allantoin in the water extract. a, allantoin (retention time: 3.253 min).

4. Discussion

Social, health, and technological developments have resulted in increases in the proportion of older people increasing worldwide along with increasing life expectancy [23]. The aging process is responsible for many changes in body composition, particularly loss of skeletal muscle mass. Muscle mass loss and dysfunction in older people are associated with various types of disease, injury or aging which significantly increases the cost of health care [24]. Age-related reductions in muscle mass known as sarcopenia induce negative effects on

muscle strength and muscle quality, as well as decreased physical function, all of which lead to mobility impairment, disability, fatigue, risk of metabolic disorders, falls, and mortality in older adults [1]. Recent research strategies have focused on factors associated with muscle mass and strength, as well as nutritional interventions; specifically, diets rich in proteins and antioxidant supplements and various exercise-related interventions are thought to increase muscle strength and physical function [23,25]. Although potent pharmaceutical treatments such as hormone therapies, angiotensin converting enzyme inhibitors and ghrelin agonists have been studied, there has been little convincing evidence of their effects or they have induced adverse side effects [25]. Nevertheless, it is necessary to find and implement interventions to prevent and treat sarcopenia in the ageing population.

Dietary supplemental herbs with many beneficial effects have long been considered to enhance health status and physical strength as well as to improve abnormal status among the elderly [26]. Yams are commonly used in medications because of their various pharmaceutical functions, which include enhancement of the digestive process in the stomach and intestines, immune regulation, and antiaging, antiinflammation and antioxidation effects. In traditional medicine, yams are known as a nourishing herb that alleviates yin deficiency in the spleen, lung, and kidney by providing a supplementary energy, qi, therefore, it is used to treat metabolic syndromes such as obesity, diabetes, and hypothyroidism [27]. In addition, yams have been used to prevent the aging process (e.g., muscle weakness) because they control muscle function by spleen control [28]. However, there is little known about the medicinal effects of yams on muscle function. In the present study, we investigated whether yam extract and its active compound, allantoin could help enhance the muscle function in myotubes. The results revealed that yam extract and allantoin significantly increased myoblast differentiation into myotubes in C2C12 cells and mitochondrial biogenesis through upregulation of the mitochondrial transcription factors, PGC1 α , TFAM, NRF1, and SIRT1 via activation of the AMPK/ACC signaling pathway.

To overcome muscle wasting in sarcopenia, it is necessary to stimulate the myogenesis pathway or inhibit the muscle wasting process. Satellite cells such as C2C12 myoblasts undergo expansion and migration and differentiate into multinucleated fibers, myotubes via myoblasts fusion [29]. Myoblast differentiation is orchestrated by myogenic regulatory factors (MRFs) such as MyoD, MRF4, Myf5, and myogenin [29]. Mature

myotubes also express structural muscle proteins such as tropomyosin and MyHC, which is the motor protein of muscle thick filaments and a specific mature marker protein [30]. In the present study, yam extract and allantoin significantly increased the expression of MyHC mRNA and protein in C2C12 myotubes, suggesting that yams and allantoin can facilitate myoblast differentiation in muscle cells; however, future investigations of the regulation of MRFs and their signals are still needed to better understand the effects of yams and allantoin on myogenesis.

Skeletal muscle, which is a key tissue involved in control of energy metabolism, processes up to 75% of insulin-stimulated glucose disposal by the translocation of GLUT4 to the plasma membrane in response to activation or resulting in activation of the AMPK pathway [31]. AMPK is a key energy sensor controlling metabolic homeostasis at both the cellular and whole-body levels and is therefore the subject of several studies of obesity, diabetes, and metabolic syndromes [32]. In myoblast differentiation, cellular ATP consumption elevates cellular AMP/ATP ratio which stimulates ATP generation through AMPK activation [32,33]. AMPK activation mediates increased the expression of GLUT-4 and mitochondrial biogenesis and regulates fatty acid oxidation via the phosphorylation of ACC and the expression of Sirt1 [34]. Sirt1 is another downstream regulator of glucose and lipid metabolism that is known to improve insulin sensitivity and to stimulate mitochondrial biogenesis in skeletal muscle via interaction with AMPK/PGC1 α [35]. PGC1 α is a major regulator of mitochondrial biogenesis that activates the expression of its downstream transcription factors, NRF-1 and TFAM [36]. In the present study, yam extract and allantoin significantly increased the levels of glucose uptake and ATP in the myotubes through upregulation of the mitochondrial biogenesis regulating factors PGC1 α , SIRT1, NRF-1, and TFAM, as well as through activation of the AMPK/ACC pathway. These results indicate that yam extract and allantoin can help elevate the mitochondrial biogenesis in skeletal muscle.

In *Dioscorea* species, the rhizomes of *D. batatas*, *D. opposita*, and *D. japonica* are commonly used as cultivated edible yams, but many wild varieties have rhizomes with different tastes and are not generally edible. However, it was reported batatasin IV, raspberry ketone, 2-methoxy-4'-hydroxyacetophenone, (3R,5S)-3,5-dihydroxy-1,7-bis(4-hydroxy-3-methoxyphenyl) heptane, β -sitosterol, blumenol A, dihydropinosylvin, stilbostemin N, butyl- β -D-fructofuranoside, allantoin, dioscin, and coreajaponins A(1) and B(2) in 50% EtOH

extract of *D. japonica* [37], steroidal saponins such as protodioscin, dioscin, and gracilin in CH₃CN extract of in *D. tokoro* (wild yam) [17], and batatasin I and 6,7-dihydroxy-2,4-dimethoxy phenanthrene in MeOH extract of *D. batatas* aerial bulbil [38]. Allantoin was identified in the water extract of flesh and peel of *D. opposite* by HPLC-PAD [39]. In our analysis, it was found allantoin (0.36 mg/mL) in the water extract of *D. batatas*.

Allantoin is the main active compound in yam and has been reported to have antidiabetic effects with plasma glucose-lowering action and glucose uptake-enhancing action in muscle tissue [11,18,40]. Under diabetic conditions including insulin resistance, hyperglycemia is a risk factor for age-related loss of muscle mass in sarcopenia, which induces muscle synthesis reduction, chronic inflammation, and mitochondrial dysfunction [41]. Therefore, we will investigate the effects of yam extract and allantoin in muscle dysfunction under diabetic conditions and evaluate its applicability as a natural source for food materials and medication for the treatment of sarcopenia in future studies.

5. Conclusions

In this study, yam water extract and its active compound, allantoin significantly improved C2C12 myoblast differentiation into myotubes by increasing the MyHC expression. In addition, these compounds significantly increased the glucose uptake and ATP production in myotubes through upregulation of the mitochondrial biogenesis-regulating factors PGC1 α , NRF1, TFAM, and Sirt1 and activation of the AMPK/ACC signaling pathway. Our results suggest that yams and allantoin can help prevent age-related muscle dysfunction and are applicable for use as anti-sarcopenic agents.

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Authors' contributions: YK Park and HW Jung designed the study. JN Ma, SY Kang, JH Park and HW Jung performed the experiment and conducted statistical analysis. JN Ma and HW Jung wrote the manuscript. All authors revised the manuscript and approved the

final version.

Conflicts of Interest: The authors declare that they have no conflicts of interest.

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