Effects of American wild ginseng and Korean cultivated wild ginseng pharmacopuncture extracts on the regulation of C2C12 myoblasts differentiation through AMPK and PI3K/Akt/mTOR signaling pathway

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Abstract. Targeting impaired myogenesis and mitochondrial biogenesis offers a potential alternative strategy for balancing energy to fight muscle disorders such as sarcopenia. In traditional Korean medicine, it is believed that the herb wild ginseng can help restore energy to the elderly. The present study investigated whether American wild ginseng pharmacopuncture (AWGP) and Korean cultivated wild ginseng pharmacopuncture (KCWGP) regulate energy metabolism in skeletal muscle cells. C2C12 mouse myoblasts were differentiated into myotubes using horse serum for 5 days. An MTT colorimetric assay verified cell viability. AWGP, KCWGP (0.5, 1, or 2 mg/ml), or metformin (2.5 mM) for reference were used to treat the C2C12 myotubes. The expressions of differentiation and mitochondrial biogenetic factors were measured by western blotting in C2C12 myotubes. Treatment of C2C12 cells stimulated with AWGP and KCWGP at a concentration of 10 mg/ml did not affect cell viability. AWGP and KCWGP treatments resulted in significant increases in the myogenesis proteins, myosin heavy chain, myostatin, myoblast determination protein 1 and myogenin, as well as increases to the biogenic regulatory factors, peroxisome proliferator-activated receptor-γ coactivator-1-α, nuclear respiratory factor 1, mitochondrial transcription factor A and Sirtuin 1, in the myotubes through AMPK and PI3K/AKT/mTOR signaling pathway activation. These results suggest that AWGP and KCWGP may be beneficial to muscle function by improving muscle differentiation and energy metabolism.

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Introduction

The world is experiencing aging populations regardless of development level (1). Projections suggest twice as a number of individuals >60 years by 2050, fueled by annual increases to this population (2). Population aging is a major issue with extensive consequences for society as a whole (1). As the elderly population increases, sarcopenia, the gradual reduction of muscle mass, strength and function with age (3-5), arises as a major risk factor (6).

The root of *Panax ginseng* (*Ginseng Radix*, Ginseng) is a promising herb with a number of active ingredients. Ginsenosides, or saponins, are the main active ingredients of Ginseng. These gensenosides are further categorized as protopanaxadiols and protopanaxatriols, represented by ginsenoside Rb1 and ginsenoside Rg1, respectively. Along with these ginsenosides, *P. ginseng* also contains alkaloids, polysaccharides, essential oil components and phenol and nitrogen compounds (7).

In traditional Korean medicine, it is believed that the herb wild ginseng can help restore energy to the elderly (8). Cultivated ginseng can also be used but wild ginseng is considered an improved option for several reasons. It has both higher ginsenoside Rb1 and Rg1 content (9) and, according to proteomic analysis, higher amino acid, amino acid-related enzyme and protein and derivative content (10). Reports also indicate that wild ginseng may be an antioxidant, an anti-inflammatory and an anticancer agent while also having antidiabetic properties (11,12). Due to its diverse biological properties, it may play a key role in treating diseases that develop later in life such as sarcopenia with muscle atrophy.

There are several different methods for administering ginseng in TKM. *P. Ginseng* has traditionally been made into a beverage by boiling its decoction in water for several hours (13), but solvents, such as water and alcohol, have recently been employed to concentrate the ginseng extract (14). These oral intake methods for ginseng have been proven to be clinically effective and safe (15,16). A new method for administering ginseng in TKM is through intramuscular

or intravenous injection at acupoints or non-acupoints using pharmacopuncture, a combination of acupuncture and herbal medicine. This acupuncture technique uses filtered and sterilized injections of herbal extracts obtained through various herbal medicine-dependent extraction methods, such as distillation, alcohol immersion and compression (17).

Wild ginseng pharmacopuncture (WGP) is a common clinical practice in TKM to cure fatigue and poor functionality of organs associated with disease (17). There are American wild ginseng pharmacopuncture (AWGP) and Korean cultivated wild ginseng pharmacopuncture (KCWGP) types. There have been reports confirming the effectiveness and safety of pharmacopuncture with KCWG. A review of an animal model injected with hydrocortisone acetate reported that ginseng pharmacopuncture may help prevent disease and enhance immune responses (18). In addition, there have been several studies on *P. ginseng* pharmacopuncture toxicity and animal safety (19,20). In a review of *P. ginseng* pharmacopuncture (7), it has been reported to affect the cardiovascular system and protein synthesis in humans, to be non-toxic in animals and humans in 25 case reports, to significantly improve the clinical outcomes of patients with serious conditions, including cancer and amyotrophic lateral sclerosis, to minor conditions, such as skin wrinkles and allergic rhinitis. Previous studies have also reported that muscle injuries and inflammation are affected by Panax ginseng extract (21,22). In a previous case study involving two elderly individuals, a combination acupuncture containing wild ginseng increased muscle-related metrics, such as muscle/fat ratio and body metabolic rate (23). However, studies on the muscle-related uses of WGP are rare. Moreover, the evidence for AWGP is lacking.

Therefore, to further evaluate how AWGP and KCWGP may be beneficial to muscle function, the present study investigated the regulation of myogenic and mitochondrial biogenic factors in C2C12 mouse skeletal muscle cells via activation of the AMPK and PI3K/Akt/mTOR signaling pathways post-AWGP and KCWGP treatment.

Materials and methods

Preparation of AWGP and KCWGP. Wild ginseng extract was prepared by external herbal dispensaries (EHDs) adhering to Korean Good Manufacturing Practice (K-GMP) standards (24). The AWGP extract was prepared from American wild ginseng (AWG; Woominnongsan, Chungbuk, Korea) at Namsangcheon EHD, and the KCWGP extract was prepared from EHD (Yongin, Korea) with Korean cultivated wild ginseng (KCWG; Cheonbangnongsan, Seocheon, Korea). A small piece of ~100 g (for AWG) or 120 g (for KCWG) of wild ginsengs was extracted in a distillation extractor with 1,000 ml of distilled water, filtered through a two-layer mesh and dissolved by stirring with 0.9% NaCl, titrated to pH 7.4. Each extract was re-filtered through a 0.45 μm syringe filters, sterilized and sealed in a vial (1 mg/ml).

AWGP and KCWGP extracts in vials were stored at 4˚C until use in the study.

Cell culture and treatments. Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (FBS) and a penicillin/streptomycin mix (Invitrogen; Thermo Fisher Scientific, Inc.) as supplements were used to maintain mouse C2C12 myoblasts (CRL-1772; ATCC) in 5% CO_2 incubator at 37˚C. Upon becoming confluent, the cells were transferred to a differentiation medium (DMEM supplemented with 2% horse serum; Invitrogen; Thermo Fisher Scientific, Inc.) for 5 days to induce myotube differentiation. The C2C12 myotubes underwent several possible treatments. They were either treated with AWGP extract or KCWGP extract at concentrations of 0.5, 1, or 2 mg/ml, with no treatment at all, or with 2.5 mM of metformin as a reference drug.

Cell viability assay. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay was used to verify cell viability. C2C12 myoblasts were cultured in 96-well plates (1x10⁴ cells/well) overnight in 5% CO_2 incubator at 37˚C. Then over a period of 24 h in 5% CO_2 incubator at 37˚C myoblasts received varying concentrations of either AWGP or KCWGP extract. The medium of each well was discarded after treatment and replaced with a 0.5 mg/ml MTT containing medium followed by a 4 h incubation at 37˚C. The formazan crystals were dissolved by replacing the culture medium with DMSO in equal volumes and shaking at room temperature (RT) for 15 min. Finally, a microplate reader (Asys) was used to determine well absorbance at 570 nm.

Western blot. Ice-cold lysis buffer (0.1 ml of 50 mM Tris-HCl (pH 7.2) containing 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.15 M NaCl and 1% NP-40) was used to lyse the cells, which were then centrifuged at 12,000 x g for 20 min at 4˚C. A bicinchoninic acid (BCA) assay was used to determine the protein content. Electrophoresis through 10% SDS-acrylamide gels was used to separate equal amounts of protein (20 μg/ml). An electrical transfer system was then used to relocate the proteins to nitrocellulose membranes. The membranes received a treatment of 3% skimmed milk in TBST buffer (5 mM Tris-HCl, pH 7.6, 136 mM NaCl and 0.1% Tween-20) for 1 h at RT to block non-specific binding.

The membranes underwent incubation with primary antibodies anti-peroxisome proliferator-activated receptor-γ coactivator-1-α (PGC-1α, 1:1,000; cat. no. NBP1-04676; Bioss Antibodies), anti-mitochondrial transcription factor A (TFAM, 1:1,000; cat. no. PA5-68789; Thermo Fisher Scientific, Inc.), anti-nuclear respiratory factor-1 (NRF-1, 1:1,000; cat. no. 69432s; Cell Signaling Technology, Inc.), anti-phospho-AMPK (1:500; cat. no. 44-11509; Thermo Fisher Scientific, Inc.), anti-AMPK (1:500; cat. no. AHO1332; Thermo Fisher Scientific, Inc.), anti-Sirtuin 1 (SIRT1, 1:1,000; cat. no. 69432s; Cell Signaling Technology, Inc.), anti-myosin heavy chain (MyHC, 1:1,000; cat. no. sc-376157; Santa Cruz Biotechnology, Inc.), anti-myoblast determination protein 1 (1:1,000; MyoD; cat. no. sc-377460; Santa Cruz Biotechnology, Inc.), anti-Myostatin (1:1,000; cat. no. PA5-11936, Invitrogen), anti-Myogenin (1:1,000; cat. no. sc-377460; Santa Cruz Biotechnology, Inc.), anti-phospho-AKT (1:500; cat. no. AF887; R&D Systems, Inc.), anti-AKT (1:500; cat. no. 9272s; Cell Signaling Technology, Inc.), anti-mTOR (1:500; cat. no. 29725; Cell Signaling Technology, Inc.) and anti-β-Actin (1:1,000; MilliporeSigma) overnight at 4˚C. The membranes then underwent incubation at RT for 1 h with
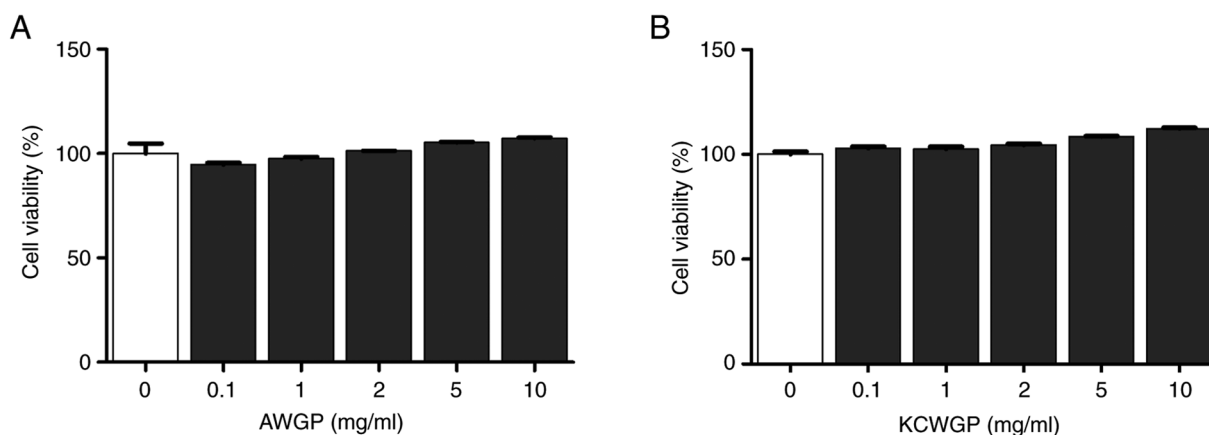
Figure 1. Cytotoxic effect of AWGP and KCWGP in C2C12 myotubes. C2C12 myoblasts received AWGP and KCWGP treatments ranging from 0.1 to 10 mg/ml for 24 h. MTT assay determined cell viability. The values from the triplicate of experiments are given as the mean ± standard error of mean. (A) AWGP. (B) KCWGP. AWGP, American wild ginseng pharmacopuncture; KCWGP, Korean cultivated wild ginseng pharmacopuncture.

horseradish peroxidase-labeled anti-mouse immunoglobulin G (IgG, 1:1,000; cat no. sc-2005; Santa Cruz Biotechnology, Inc.). Following incubation, the blots were washed in 1X TBST three times. Then ECL western detection reagents (cat no. 1705061; Bio-Rad Laboratories, Inc.) were used for development and densitometry was used to compare the protein bands in ImageJ software (1.47J, National Institutes of Health).

Immunocytochemistry. C2C12 myoblasts were cultured in DMEM with 10% FBS in 5% CO_2 at 37°C and was performed to differentiate the C2C12 myoblasts for 5 days by first seeding them onto Thermanox plastic coverslips (Nunc; Thermo Fisher Scientific, Inc.). Following drug treatment, 1X PBS was used to wash the samples on coverslips. Then 10 min treatment of 4% paraformaldehyde was used to fix them and a 20 min treatment of 0.1% Triton X-100 (MilliporeSigma) was used to permeabilize them at RT. Another rinse with 1X PBS was performed and a 30 min treatment at RT with 5% bovine serum albumin (BSA) was performed to block the coverslips followed by an overnight incubation with anti-MyHC antibody (1:200; cat. no. sc-376157; Santa Cruz Biotechnology, Inc.) at 4°C. Next, a 1 h treatment with Alexa Fluor 488-conjugated goat anti-rabbit antibody (1:500; cat. No. A11011; Thermo Fisher Scientific. Inc.) at RT was used to label the coverslips and then a 5 min DAPI treatment was used to counterstain at RT. Lastly, observation of MyHC expression was performed 24 h after drug treatment with a fluorescence microscope (Leica DM2500; Leica Microsystems GmbH).

High-performance liquid chromatography (HPLC). The AWGP and KCWGP constituents were identified via HPLC with standard compounds, rg1, rb1, rg3 and rh2. HPLC analysis was performed at a wavelength of 203 nm and a flow rate of 0.6 ml/min using Waters HPLC (Waters Corporation) as an instrument and YMC 3 μm, 4.6x150 mm (AQ; YMC Korea Co., Ltd.) as a column.

An acetonitrile (B) and water (A) gradient solvent system was used for chromatographic separation using the following gradient solvent system procedure at RT: 0 min, 20% B; 4 min, 22% B; 20 min, 33% B; 26 min, 38% B; 40 min, 38% B; 58 min, 50% B; 68 min, 50% B; 70 min, 60% B; 75 min, 60% B; 80 min, 20% B and 90 min, 20% B. The standards mixture (rg1, rb1, rg3 and rh2) was injected at a volume of 10 μl and a concentration of 0.5 mg/ml and the AWGP and KCWGP medicinal solutions were injected at a volume of 100 μl.

Data analysis. For consistency, each experiment was repeated three times with results given as mean ± standard error of mean. One-way ANOVA and a Tukey's test were performed with GraphPad Prism (GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Cytotoxic effect of AWGP and KCWGP in C2C12 cells. A cell viability assay was performed to determine the cytotoxicity of AWGP and KCWGP. The results showed that treatment of C2C12 cells stimulated with AWGP and KCWGP at a concentration of 10 mg/ml did not affect cell viability (Fig. 1). In the present study, AWGP and KCWGP were used at concentrations of 0.5, 1 and 2 mg/ml in C2C12 myotubes.

Effects of AWGP and KCWGP on myogenesis-regulating protein expression in C2C12 myotubes. AWGP and KCWGP's influence on myoblast differentiation into myotubes was observed using western blotting to detect myogenesis-regulating proteins, such as MyHC, myostatin, MyoD and myogenin, in C2C12 myotubes.

The C2C12 myotubes treated with AWGP had higher MyHC, myostatin, MyoD and myogenin protein expression compared with the untreated cells. Furthermore, the increases were proportional to the concentrations of AWGP. The C2C12 myotubes treated with KCWGP also showed significant increases in MyHC, myostatin, MyoD and myogenin protein expression compared with the untreated cells, but only the increases in MyHC and myostatin protein expression were dose-dependent.

MyHC expression increased significantly in samples treated with 1 and 2 mg/ml AWGP ($P<0.05$, respectively), 2 mg/ml KCWGP ($P<0.05$) and metformin ($P<0.001$). MyoD expression increased significantly in samples treated with 2 mg/ml AWGP ($P<0.05$), 0.5 mg/ml AWGP ($P<0.01$) and 0.5 mg/ml
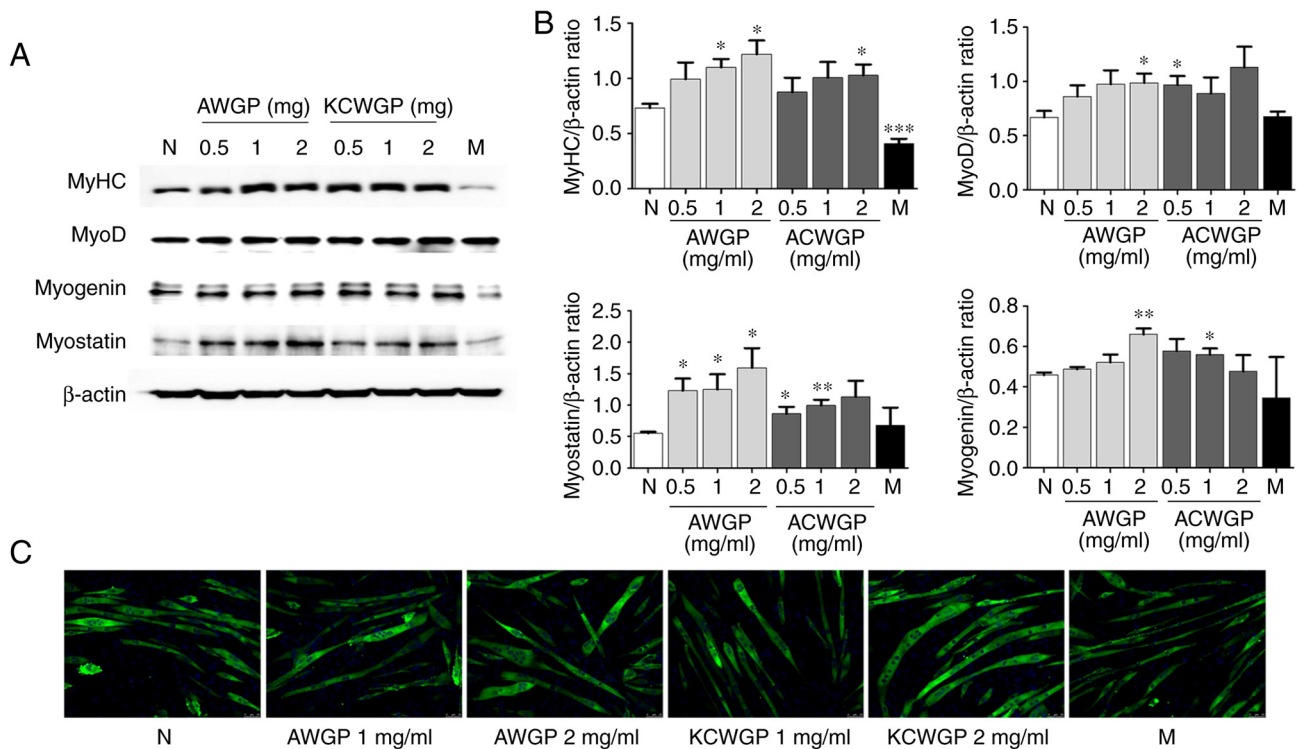
Figure 2. Effect of AWGP and KCWGP on differentiation in C2C12 myotubes. C2C12 myoblasts were differentiated into myotubes for 5 days and then treated with AWGP or KCWGP (0.1, 1 or 2 mg/ml) or with metformin (2.5 mM) for 24 h. The expression of MyHC protein was determined by (A) western blotting and (B) the density of each target was calculated with the expression of β-actin. (C) Immunocytochemical staining; fluorescence microscopy images were captured at x200 (scale bar=25 *μ*M) with MyHC-positive cells (green) and DAPI (blue). The values from the triplicate of experiments are given as the mean ± standard error of mean. *P<0.05, **P<0.01 and ***P<0.001 vs. N. AWGP, American wild ginseng pharmacopuncture; KCWGP, Korean cultivated wild ginseng pharmacopuncture; MyHC, myosin heavy chain; M, metformin-treated cells; N, untreated cells.

KCWGP (P<0.05) (Fig. 2A). Myostatin expression increased significantly in samples treated with all concentrations of AWGP (P<0.05) and 1 and 2 mg/ml KCWGP (P<0.05 and P<0.01, respectively). Lastly, myogenin expression increased significantly in the samples treated with 2 mg/ml AWGP (P<0.01) and 1 mg/ml KCWGP (P<0.05) (Fig. 2B).

The immunocytochemical staining (Fig. 2C) showed that C2C12 myotubes differentiated into long, wide cylinders with multiple nuclei due to an increase in MyHC expression following treatment with AWGP or KCWGP. This change occurred in a dose-dependent manner. Metformin-treated cells showed an increase in MyHC expression, but less than in the treatment of AWGP and KCWGP. Therefore, it appears that muscle differentiation stimulation may be aided by AWGP and KCWGP (Fig. 2C).

Effects of AWGP and KCWGP on mitochondrial biogenesis-regulating protein expression in C2C12 myotubes. The influence of AWGP and KCWGP on muscle cell biogenesis in C2C12 myotubes was confirmed using western blotting to observe the expression of the mitochondrial biogenesis regulators, PGC-1α, NRF-1, TFAM and Sirt-1. C2C12 myotubes samples that were treated with either AWGP or KCWGP had higher expression of all four regulatory proteins compared with the untreated cells. PGC-1α expression increased significantly in samples treated with every experimental dosage of both AWGP (P<0.05) and KCWGP (P<0.05). Sirt-1 expression increased significantly in samples treated with 2 mg/ml AWGP

(P<0.05) and 1 mg/ml KCWGP (P<0.05). NRF-1 expression increased significantly only in samples treated with 0.5 and 1 mg/ml KCWGP (P<0.05). Finally, TFAM expression increased significantly in samples treated with 1 mg/ml AWGP (P<0.05) and all experimental dosages of KCWGP (P<0.05). As these four regulatory proteins all showed increased expression with AWGP and KCWGP treatments, these treatments may be involved in upregulating transcription factors to improve mitochondrial biogenesis (Fig. 3).

Effects of AWGP and KCWGP on the AMPK and PI3K/AKT/mTOR pathways in C2C12 myotubes. The influence of AWGP and KCWGP on the AMPK and PI3K/Akt/mTOR signaling pathways, which activate mitochondrial biogenesis in C2C12 myotubes, was investigated.

Myotubes treated with AWGP increased the expression of PI3K (Fig. 4B) and phosphorylation of AMPK (Fig. 4A), Akt (Fig. 4B) and mTOR (Fig. 4C) compared with the untreated cells in a dose-dependent manner. The treatment of KCWGP treatment increased the expression of PI3K (Fig. 4B) and phosphorylation of AMPK (Fig. 4A), Akt (Fig. 4B) and mTOR (Fig. 4C) compared with the untreated cells and there was little difference between the KCWGP concentrations.

AMPK phosphorylation increased significantly in samples treated with 1 mg/ml AWGP (P<0.01), 2 mg/ml AWGP (P<0.001), 0.5 mg/ml KCWGP (P<0.01), 1 mg/ml KCWGP (P<0.05), 2 mg/ml KCWGP (P<0.01) and metformin (P<0.01). Akt phosphorylation increased significantly when samples
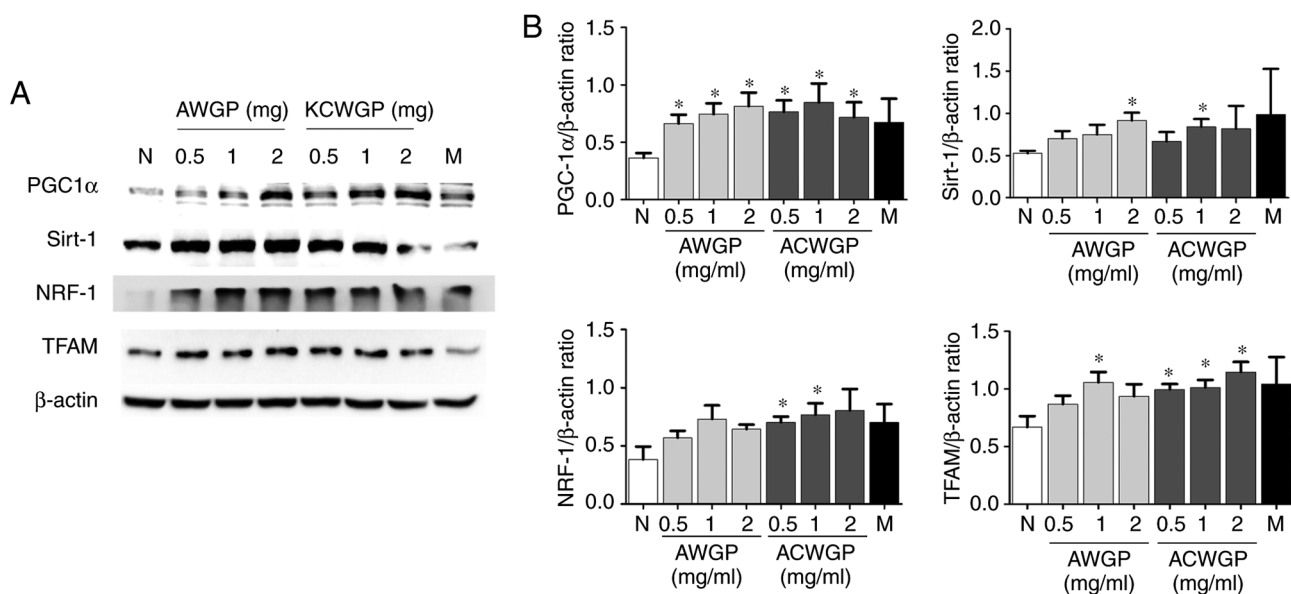
Figure 3. Effect of AWGP and KCWGP on mitochondrial biogenesis in C2C12 myotubes. C2C12 myoblasts were differentiated into myotubes for 5 days and then treated with AWGP or KCWGP (0.1, 1 or 2 mg/ml) or with metformin (2.5 mM) for 45 min. (A) The expressions of PGC1α, Sirt-1, NRF-1 and TFAM proteins were analyzed by western blotting. (B) The density of each target was calculated with the expression of β-actin. The values from the triplicate of experiments are given as the mean ± standard error of mean. *P<0.05 vs. untreated cells (N). AWGP, American wild ginseng pharmacopuncture; KCWGP, Korean cultivated wild ginseng pharmacopuncture; PGC1α, peroxisome proliferator-activated receptor-γ coactivator-1-α; Sirt-1, Sirtuin 1; NRF-1, nuclear respiratory factor-1; TFAM, mitochondrial transcription factor A; M, metformin-treated cells; N, untreated cells.

were treated with 0.5 mg/ml AWGP (P<0.05), 1 mg/ml AWGP (P<0.01), 2 mg/ml AWGP (P<0.01), all concentrations of KCWGP (P<0.05) and metformin (P<0.01). PI3K expression increased significantly when samples were treated with all experimental concentrated of both AWGP and KCWGP (P<0.05) as well as when treated with metformin (P<0.01). mTOR phosphorylation increased significantly when samples were treated with all experimental concentrations of AWGP (P<0.01) and KCWGP (P<0.05). These increases in phosphorylation imply AWGP and KCWGP may aid in activating the AMPK and PI3K/AKT/mTOR pathways and enhancing mitochondrial biogenesis in C2C12 myotubes.

HPLC analysis of AWGP and KCWGP. To identify the constituents of AWGP and KCWGP, HPLC analysis was performed with standard compounds, rg1, rb1, rg3 and rh2 (Fig. 5A).

In AWGP (Fig. 5B) and KCWGP (Fig. 5C), the expected peaks from rb1, rg1 and rg3 were found, but not the expected peak from rh2. In the case of KCWGP, a small peak, which was considered to be an rg3 component, was observed and the rg3 component is expected to be contained in a trace amount.

Discussion

The regulation of carbohydrate metabolism and the balancing of energy in the body given proper nutrition is achieved in skeletal muscles (25). Within these muscles are mitochondria, which not only supply ATP as cellular energy (26), but also can aid in the metabolism of amino acid and the homeostasis of ions (27). As mitochondria have diverse roles in moving the myoblasts from the glycolytic state in muscles (28), the upregulation of mitochondrial functions appear to promote myoblast differentiation and muscle functions (29).

The C2C12 myoblast cell line is often used for investigating skeletal muscle atrophy through *in vitro* modeling (30,31). This cell line can easily differentiate into myotubes in low-serum medium through the regulation of the myogenic differentiation gene (MyoD) transcription factor family (32), which includes the myogenic regulatory factors (MRFs), myogenic factor 5 (Myf5), MyoD and myogenin (32). MRF activation causes myoblasts to fuse into myotubes (32). Below the basal membrane of muscle tissue are the inactive skeletal muscle-derived stem cells required for myogenesis (33). Upon activation, they begin myogenic differentiation and proceed through a cascade of MRFs and structural muscle proteins, including MyHC, which is a muscle thick filament motor protein that can be used to indicate maturation (34,35). Thus, MRFs activation causes myoblasts to fuse into myotubes. First, Myf5 is initially expressed after satellite cells are activated and then MyoD and myogenin are sequentially expressed in the newly formed fibers (33). However, in the present study, treatments of AWGP and KCWGP extracts to C2C12 myotubes significantly increased MyHC, myostatin, MyoD and myogenin expression resulting in changes in myotube morphology. This increased expression implies that myoblast differentiation into myotubes in muscles can be stimulated with AWGP and KCWGP. Since the expression of MyoD, myogenin, MyHC and myostatin increased after treatment with AWGP and KCWGP, these treatments may help stimulate myoblast differentiation into myotubes in muscle cells.

Metformin, one of the biguanide drugs, is a well-known medicine for the treatment of type 2 diabetes as an anti-hyperglycemic and insulin sensitizing agent. It has been reported that metformin treatment in C2C12 cells increases the myotube diameter at 48 h during differentiation (36). In the present study, metformin decreased MyHC expression in C2C12 myotubes and showed
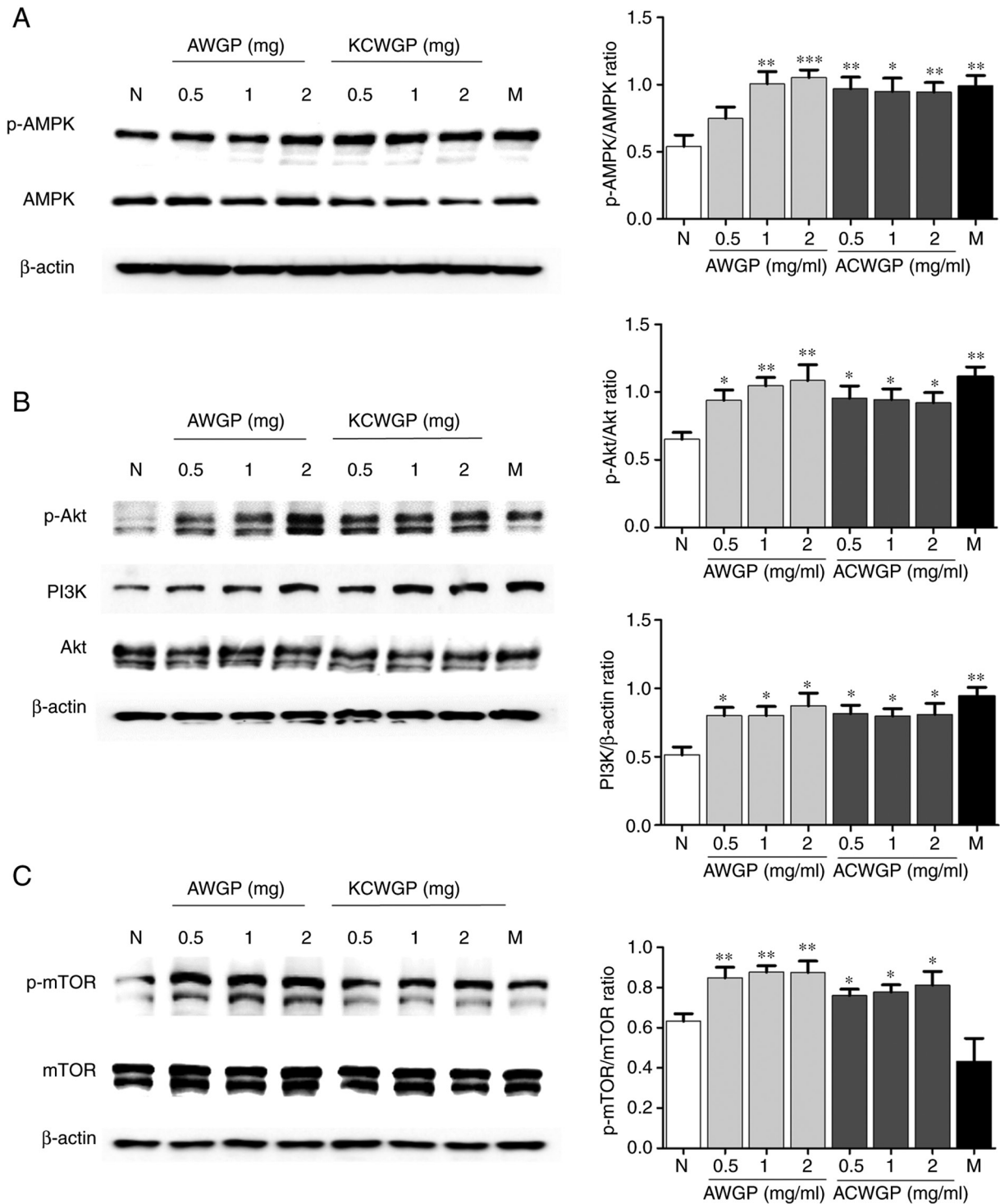
Figure 4. Effect of AWGP and KCWGP on the pathways of AMPK and PI3K/Akt/mTOR in C2C12 myotubes. C2C12 myotubes were treated with AWGP or KCWGP (0.1, 1 and 2 mg/ml) or with metformin (2.5 mM) for 45 min. Phosphorylation of (A) AMPK, (B) Akt and (C) mTOR and (B) the expression of PI3K were analyzed by western blotting. The density of each target was calculated with the expression of β-actin. The values from the triplicate of experiments are given as the mean ± standard error of mean. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ vs. untreated cells (N). AWGP, American wild ginseng pharmacopuncture; KCWGP, Korean cultivated wild ginseng pharmacopuncture; and M, metformin-treated cells; N, untreated cells.

differentially altered morphology with a longer and thinner myotube compared with AWGP and KCWGP-treated cells. However, further study is needed on the differences between AWGP or KCWGP and metformin to myoblast differentiation.

The PGC1α/SIRT1/AMPK pathways are integral to mitochondrial biogenesis and the metabolism of glucose in skeletal muscle (37-39). A number of natural compounds have been

reported to increase mitochondrial biosynthesis and oxidative phosphorylation through activation of PGC1α, AMPK and SIRT1, thereby increasing energy expenditure in skeletal muscle (40,41). PGC1α increases the rate of skeletal muscle respiration and mitochondrial biogenesis, thereby increasing the consumption of energy (42) and it also interacts with NRF-1, a transcription factor for a number of mitochondrial
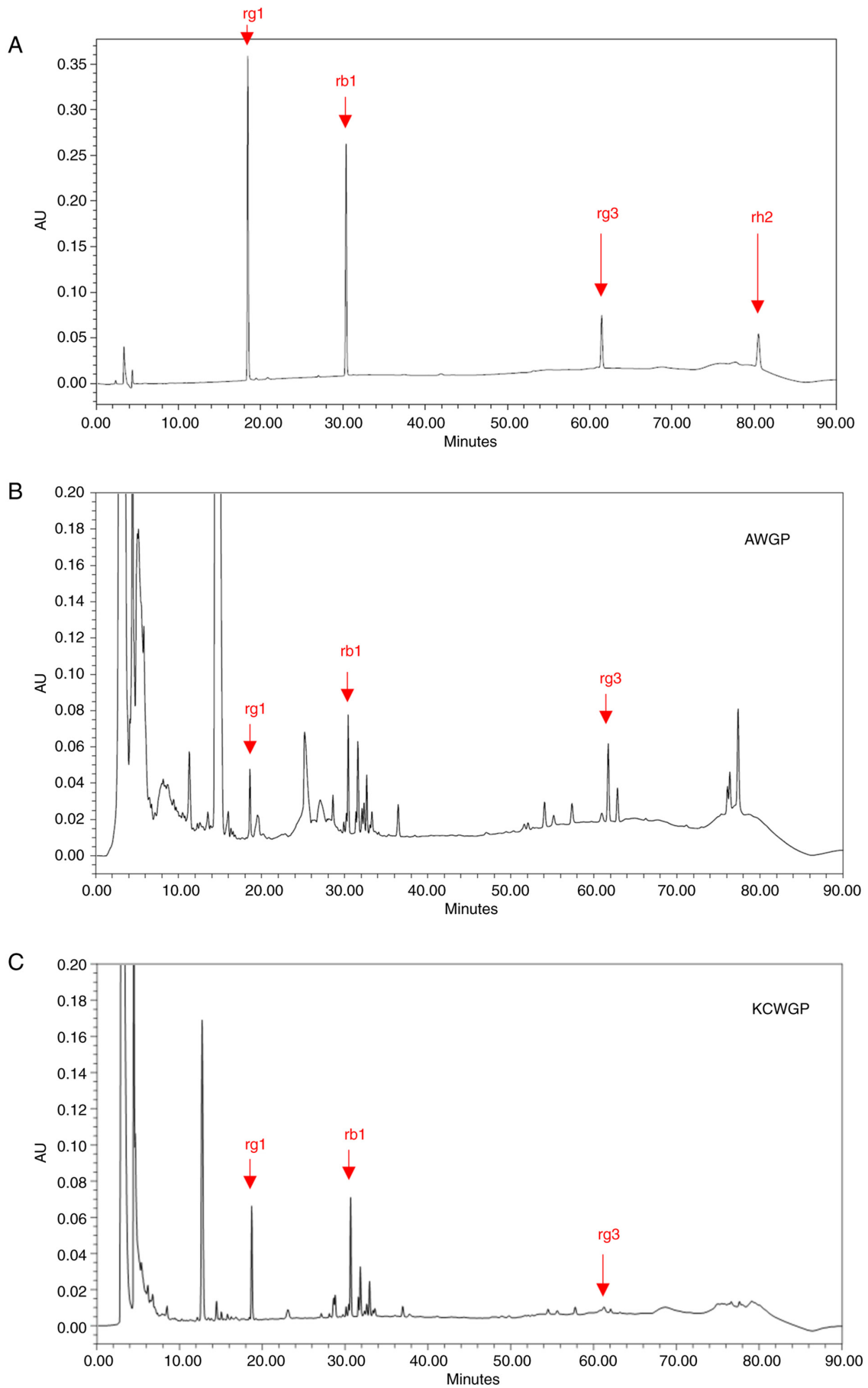
Figure 5. HPLC analysis of AWGP and KCWGP. (A) rg1, rb1, rg3 and rh2 as a standard compound. (B) rg1, rb1, rg3 and rh2 in AWGP and (C) in KCWGP. HPLC, high-performance liquid chromatography; AWGP, American wild ginseng pharmacopuncture and KCWGP, Korean cultivated wild ginseng pharmacopuncture; AU, arbitrary units.

genes, including TFAM, a direct regulator of mitochondrial DNA replication and transcription (43). In the present study, PGC1α, NRF-1 and TFAM increased according to the AWGP and KCWGP treatment concentrations in C2C12 myotubes, but the change of mitochondrial quantity such as mitochondrial DNA levels by treatment of AWGP and KCWGP should be further identified to advance the understanding of the role of drugs on energy metabolism in muscle.

By providing energy as ATP to cells, mitochondrial biogenesis ultimately promotes AMPK activation (44). It has been reported that in skeletal muscle, AMPK, p38 MAPK and AKT activate PGC-1α by changing the phosphorylation status of residues at distinct sites in the protein (45). AMPK is excessively involved in the metabolic condition of skeletal muscle through its regulation of a number of downstream targets such as the PI3K/AKT pathway (46). Due to their effects on anabolic and catabolic cellular processes, AMPK serves a major role in controlling skeletal muscle development and growth (46) and in regulating muscle mass and regeneration (47). AKT has been known to be an important regulator of both glucose transport (48,49) and activation of glycogen synthesis in skeletal muscle (50). mTOR is the main regulator in maintaining muscle mass through protein synthesis as it controls the balance between metabolic and catabolic processes. Therefore, the PI3K/Akt/mTOR pathway is important for muscle maintenance in aged muscles (51). In aging-associated conditions, it has been reported that ginseng and its bioactive compounds, ginsenoside Rb1 and Rg3, have anti-aging effects with the molecular mechanisms such as PPAR-α, GLUTs, FOXO1, caspase-3 and Bcl-2 along with SIRT1/AMPK, PI3K/Akt, NF-κB and insulin/insulin-like growth factor-1 pathways as preferential targets (52). Since the AWGP and KCWGP treatments activated the AMPK and PI3K/Akt/mTOR signaling pathways, they may increase mitochondrial biogenesis in skeletal muscle cells, thereby aiding in muscle maintenance in aged muscles.

In the present study, peaks predicted to be rb1, rg1 and rg3 were found in AWGP and KCWGP, but peaks predicted to be rh2 were not found. KCWGP was reported to contain both ginsenoside Rg1 and ginsenoside Rb2 as well as phenolic compounds while having extraction-dependent variance in the amounts of each substance (53). American ginseng (*P. quinquefolium*) is reported to have the most ginsenoside Rb1 overall, followed by Rg1 and Re (54). Tanaka (55) found that the difference in ginsenoside content in wild and cultivated Asian ginseng was insignificant, but Mizuno *et al* (56) reported that wild ginseng had higher ginsenoside Rg1, Re and Rd content and lower ginsenoside Rc, Rb2 and Rb1 content compared with the cultivated roots of Asian ginseng (*P. ginseng*). Other studies report positive effects of rg1, rb1, rg3 and/or rh2 on myogenic differentiation and myotubes formation by activating the Akt signaling pathway that has a protective function in muscle weakness and atrophy with chronic diseases and cancers (57-60). The HPLC analysis identified rg1, rb1 and rg3 in AWGP and KCWGP. However, because there is a large diversity of ginsenosides in AWGP, analysis of various compounds will need to be further studied.

In the present study, AWGP and KCWGP were investigated for their regulation of muscle differentiation and mitochondrial biogenesis via the AMPK and PI3K/AKT/mTOR signaling pathways in C2C12 myotubes. AWGP and KCWGP significantly increased the expression of MyoD, myogenin, MyHC and myostatin and increased the expression of the PGC-1α, NRF-1, TFAM and SIRT1 in the myotubes through activation of the AMPK and PI3K/Akt/mTOR signaling pathway. The results suggested that AWGP and KCWGP may aid muscle function through muscle differentiation and energy metabolism enhancement. However, to clearly understand the mechanisms of AWGP and KCWGP for the regulation of muscle differentiation and mitochondrial biogenesis, further studies on the pathological conditions such as energy metabolism imbalance will be needed.

Availability of data and materials

The data generated and analyzed during the study are available from the corresponding author upon reasonable request. All materials used in this study are properly included in the Methods section.

Authors' contributions

HWJ and JHH designed all of the experiments together. HWJ, SYK and JHH performed the experiments, the statistical analysis and interpreted the experimental results. SYK and JHH wrote the manuscript. HWJ revised the manuscript. All authors have read and approved the final manuscript. HWJ, SYK and JHH confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. United Nations Population Fund (UNFPA) and HelpAge International: Ageing in the twenty-first century: A celebration and a challenge. https://www.unfpa.org/publications/ageing-twentyfirst-century. Accessed January 1, 2012.
2. United Nations: World Population Prospects: The 2002 revision, highlights. United Nations, New York, NY, 2003. https://www.un.org/development/desa/pd/sites/www.un.org.development.desa.pd/files/files/documents/2020/Jan/un_2002_world_population_prospects-2002_revision_volume-ii.pdf. Accessed February 26, 2003.

3. Kalyani RR, Corriere M and Ferrucci L: Age-related and disease-related muscle loss: The effect of diabetes, obesity, and other diseases. Lancet Diabetes Endocrinol 2: 819-829, 2014.
4. Dodds R and Avan AA: Sarcopenia and frailty: New challenges for clinical practice. Clin Med (Lond) 16: 455-458, 2016.
5. Cruz-Jentoft AJ, Baeyens JP, Bauer JM, Boirie Y, Cederholm T, Landi F, Martin FC, Michel JP, Rolland Y, Schneider SM, et al: Sarcopenia: European consensus on definition and diagnosis: Report of the European working group on sarcopenia in older people. Age Ageing 39: 412-423, 2010.
6. Kamel HK: Sarcopenia and aging. Nutr Rev 61: 157-167, 2003.
7. Lee IS, Kang KS and Kim SY: Panax ginseng pharmaco-puncture: Current status of the research and future challenges. Biomolecules 10: 33, 2019.
8. Park SY, Park JH, Kim HS, Lee CY, Lee H.J, Kang KS and Kim CE: Systems-level mechanisms of action of Panax ginseng: A network pharmacological approach. J Ginseng Res 42: 98-106, 2018.
9. Jeong HS, Lim CS, Cha BC, Choi SH and Kwon KR: Component analysis of cultivated ginseng, cultivated wild ginseng, and wild ginseng and the change of ginsenoside components in the process of red ginseng. J Pharmacopuncture 13: 63-77, 2010.
10. Sun H, Liu F, Sun L, Liu J, Wang M, Chen X, Xu X, Ma R, Feng K and Jiang R: Proteomic analysis of amino acid metabolism differences between wild and cultivated Panax ginseng. J Ginseng Res 40: 113-120, 2016.
11. Yang Y, Yang WS, Yu T, Sung GH, Park KW, Yoon K, Son YJ, Hwang H, Kwak YS, Lee CM, et al: ATF-2/CREB/IRF-3-targeted anti-inflammatory activity of Korean red ginseng water extract. J Ethnopharmacol 154: 218-228, 2014.
12. Lee CH and Kim JH: A review on the medicinal potentials of ginseng and ginsenosides on cardiovascular diseases. J Ginseng Res 38: 161-166, 2014.
13. Sung IJ, Ghimeray AK, Chang KJ, and Park CH: Changes in contents of Ginsenoside due to boiling process of Panax ginseng C.A. Mayer. Korea J Plant Res 26: 726-730, 2013.
14. Lee SM, Bae BS, Park HW, Ahn NG, Cho BG, Cho YL and Kwak YS: Characterization of Korean Red Ginseng (Panax ginseng Meyer): History, preparation method, and chemical composition. J Ginseng Res 39: 384-391, 2015.
15. Jin X, Che DB, Zhang ZH, Yan HM, Jia ZY and Jia XB: Ginseng consumption and risk of cancer: A meta-analysis. J Ginseng Res 40: 269-277, 2016.
16. Hernández-García D, Granado-Serrano AB, Martín-Gari M, Naudí A and Serrano JC: Efficacy of Panax ginseng supplementation on blood lipid profile. A meta-analysis and systematic review of clinical randomized trials. J Ethnopharmacol 243: 112090, 2019.
17. Jung C, Jung JH and Lee MS: A clinical study of immune pharmacopuncturology. Kyungrak Medical Publishing Co., pp127-133, Chungnam, 2011 (In Korean).
18. Kang SK, Lee HJ and Park YB: Experimental studies on the effect of Ginseng radix aqua-acupuncture. Int Symp East-West Med 1989: 61-83, 1989.
19. Lim C, Kwon K and Lee K: Plexiform neurofibroma treated with pharmacopuncture. J Pharmacopuncture 17: 74-77, 2014.
20. Lee K, Yu J, Sun S, Kwon K and Lim C: A 4-week, repeated, intravenous dose, toxicity test of mountain ginseng pharmaco-puncture in sprague-dawley rats. J Pharmacopuncture 17: 27-35, 2014.
21. Jung HL, Kwak HE, Kim SS, Kim YC, Lee CD, Byurn HK and Kang HY: Effects of Panax ginseng supplementation on muscle damage and inflammation after uphill treadmill running in humans. Am J Chin Med 39: 441-450, 2011.
22. Alvarez AI, De Oliveira ACC, Perez AC, Vila L, Ferrando A and Prieto JG: The effect of ginseng on muscle injury and inflammation. J Ginseng Res 28: 18-26, 2004.
23. Hwang JH and Jung HW: Effects of pharmacopuncture with wild ginseng complex in 2 elderly patients with obesity: Case report. Medicine (Baltimore) 97: e11534, 2018.
24. Sung SH, Shin BC, Park MJ, Kim KH, Kim JW, Ryu JY and Park JK: Current status of management on pharmacopuncture in Korea through introduction of an accreditation system. J Pharmacopuncture 22: 75-82, 2019.
25. Papa EV, Dong X and Hassan M: Skeletal muscle function deficits in the elderly: Current perspectives on resistance training. J Nat Sci 3: e272, 2017.
26. Kelly DP and Scarpulla RC: Transcriptional regulatory circuits controlling mitochondrial biogenesis and function. Genes Dev 18: 357-368, 2004.
27. Hock MB and Kralli A: Transcriptional control of mitochondrial biogenesis and function. Annu Rev Physiol 71: 177-203, 2009.
28. Sin J, Andres AM, Taylor DJ, Weston T, Hiraumi Y, Stotland A, Kim BJ, Hwang C, Doran KS, and Gottlieb RA: Mitophagy is required for mitochondrial biogenesis and myogenic differentiation of C2C12 myoblasts. Autophagy 12: 369-380, 2016.
29. Oh SS, Kim S, Moon S, Park DH and Kang JH: Lactate overload inhibits myogenic activity in C2C12 myotubes. Open Life Sci 14: 29-37, 2019.
30. Shen H, Liu T, Fu L, Zhao S, Fan B, Cao J and Li X: Identification of microRNAs involved in dexamethasone-induced muscle atrophy. Mol Cell Biochem 381: 105-113, 2013.
31. Wang DT, Yin Y, Yang YJ, Lv PJ, Shi Y, Lu L and Wei LB: Resveratrol prevents TNF-α-induced muscle atrophy via regulation of Akt/mTOR/FoxO1 signaling in C2C12 myotubes. Int Immunopharmacol 19: 206-213, 2014.
32. Langley B, Thomas M, Bishop A, Sharma M, Gilmour S and Kambadur R: Myostatin inhibits myoblast differentiation by down-regulating MyoD expression. J Biol Chem 277: 49831-49840, 2002.
33. Tsukamoto S, Shibasaki A, Naka A, Saito H and Iida K: Lactate promotes myoblast differentiation and myotube hypertrophy via a pathway involving MyoD in vitro and enhances muscle regeneration in vivo. Int J Mol Sci 19: 3649, 2018.
34. Cole NJ, Hall TE, Martin CI, Chapman MA, Kobiyama A, Nihei Y, Watabe S and Johnston IA: Temperature and the expression of myogenic regulatory factors (MRFs) and myosin heavy chain isoforms during embryogenesis in the common carp Cyprinus carpio L. J Exp Biol 207: 4239-4248, 2004.
35. Tajbakhsh S: Skeletal muscle stem cells in developmental versus regenerative myogenesis. J Intern Med 266: 372-389, 2009.
36. Crocker CL, Baumgarner BL and Kinsey ST: β-guanidinopropionic acid and metformin differentially impact autophagy, mitochondria and cellular morphology in developing C2C12 muscle cells. J Muscle Res Cell Motil 41: 221-237, 2020.
37. Cantó C and Auwerx J: PGC-1alpha, SIRT1 and AMPK, an energy sensing network that controls energy expenditure. Curr Opin Lipidol 20: 98-105, 2009.
38. Cantó C, Gerhart-Hines Z, Feige JN, Lagouge M, Noriega L, Milne JC, Elliott PJ, Puigserver P and Auwerx J: AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity. Nature 458: 1056-1060, 2009.
39. Ruderman NB, Xu XJ, Nelson L, Cacicedo JM, Saha AK, Lan F and Ido Y: AMPK and SIRT1: A long-standing partnership? Am J Physiol Endocrinol Metab 298: E751-E760, 2010.
40. Suwa M, Egashira T, Nakano H, Sasaki H and Kumagai S: Metformin increases the PGC-1alpha protein and oxidative enzyme activities possibly via AMPK phosphorylation in skeletal muscle in vivo. J Appl Physiol (1985) 101: 1685-1692, 2006.
41. Lagouge M, Argmann C, Gerhart-Hines Z, Meziane H, Lerin C, Daussin F, Messadeq N, Milne J, Lambert P, Elliott P, et al: Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. Cell 127: 1109-1122, 2006.
42. Liang H and Ward WF: PGC-1alpha: A key regulator of energy metabolism. Adv Physiol Educ 30: 145-151, 2006.
43. Scarpulla RC: Transcriptional paradigms in mammalian mitochondrial biogenesis and function. Physiol Rev 88: 611-638, 2008.
44. Banerjee J, Bruckbauer A and Zemel MB: Activation of the AMPK/Sirt1 pathway by a leucine-metformin combination increases insulin sensitivity in skeletal muscle, and stimulates glucose and lipid metabolism and increases life span in Caenorhabditis elegans. Metabolism 65: 1679-1691, 2016.
45. Fernandez-Marcos PJ and Auwerx J: Regulation of PGC-1α, a nodal regulator of mitochondrial biogenesis. Am J Clin Nutr 93: 884S-890S, 2011.
46. Thomson DM: The role of AMPK in the regulation of skeletal muscle size, hypertrophy, and regeneration. Int J Mol Sci 19: 3125, 2018.
47. Tao R, Gong J, Luo X, Zang M, Guo W, Wen R and Luo Z: AMPK exerts dual regulatory effects on the PI3K pathway. J Mol Signal 5: 1, 2010.
48. Cho H, Mu J, Kim JK, Thorvaldsen JL, Chu Q, Crenshaw EB III, Kaestner KH, Bartolomei MS, Shulman GI and Birnbaum MJ: Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). Science 292: 1728-1731, 2001.
49. McCurdy CE and Cartee GD: Akt2 is essential for the full effect of calorie restriction on insulin-stimulated glucose uptake in skeletal muscle. Diabetes 54: 1349-1356, 2005.

50. Friedrichsen M, Birk JB, Richter EA, Ribel-Madsen R, Pehmøller C, Hansen BF, Beck-Nielsen H, Hirshman MF, Goodyear LJ, Vaag A, *et al*: Akt2 influences glycogen synthase activity in human skeletal muscle through regulation of NH2-terminal (sites 2 + 2a) phosphorylation. Am J Physiol Endocrinol Metab 304: E631-E639, 2013.
51. Yoon MS: mTOR as a key regulator in maintaining skeletal muscle mass. Front Physiol 8: 788, 2017.
52. Bai L, Gao J, Wei F, Zhao J, Wang D and Wei J: Therapeutic potential of ginsenosides as an adjuvant treatment for diabetes. Front Pharmacol 9: 423, 2018.
53. Lee DY, Choi BS, Lee IH, Kim JH and Gwon PS: Comparison of index compounds content and antioxidative activity of wild ginseng pharmacopuncture by extraction method. J Intern Korean Med 39: 313-322, 2018.
54. Lim W, Mudge KW and Vermeylen F: Effects of population, age, and cultivation methods on ginsenoside content of wild American ginseng (Panax quinquefolium). J Agric Food Chem 53: 8498-8505, 2005.
55. Tanaka O: Solubilizing properties of ginseng saponins. Soc Korean Ginseng, pp67-74, 1987.
56. Mizuno M, Yamada J, Terai H, Kozukue N, Lee YS and Tsuchida H: Differences in immunomodulating effects between wild and cultured Panax ginseng. Biochem Biophys Res Commun 200: 1672-1678, 1994.
57. Go GY, Jo A, Seo DW, Kim WY, Kim YK, So EY, Chen Q, Kang JS, Bae GU and Lee SJ: Ginsenoside Rb1 and Rb2 upregulate Akt/mTOR signaling-mediated muscular hypertrophy and myoblast differentiation. J Ginseng Res 44: 435-441, 2020.
58. Go GY, Lee SJ, Jo A, Lee J, Seo DW, Kang JS, Kim SK, Kim SN, Kim YK and Bae GU: Ginsenoside Rg1 from Panax ginseng enhances myoblast differentiation and myotube growth. J Ginseng Res 41: 608-614, 2017.
59. Lee SJ, Bae JH, Lee H, Lee H, Park J, Kang JS and Bae GU: Ginsenoside Rg3 upregulates myotube formation and mitochondrial function, thereby protecting myotube atrophy induced by tumor necrosis factor-alpha. J Ethnopharmacol 242: 112054, 2019.
60. Lee SJ, Im M, Park SK, Kim JY, So EY, Liang OD, Kang JS and Bae GU: BST204, a Rg3 and Rh2 enriched ginseng extract, upregulates myotube formation and mitochondrial function in TNF-α-induced atrophic myotubes. Am J Chin Med 48: 631-650, 2020.