# Anti-fatigue activity of polysaccharides from the fruits of four Tibetan plateau indigenous medicinal plants

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## Abstract

### Ethnopharmacological relevance

The fruits of *Hippophae rhamnoides* L., *Lycium barbarum* L., *Lycium ruthenicum* Murr. and *Nitraria tangutorum* Bobr. are traditional medicinal food of Tibetans and used to alleviate fatigue caused by oxygen deficiency for thousands of years. The present study focused on exploiting natural polysaccharides with remarkable anti-fatigue activity from the four Qinghai-Tibet plateau characteristic berries.

### Materials and methods

The fruits of *Hippophae rhamnoides*, *Lycium barbarum*, *Lycium ruthenicum* and *Nitraria tangutorum* were collected from Haixi national municipality of Mongol and Tibetan (N 36.32°, E98.11°; altitude: 3100 m), Qinghai, China. Their polysaccharides (HRWP, LBWP, LRWP and NTWP) were isolated by hot-water extraction, and purified by DEAE-Cellulose ion-exchange chromatography. The total carbohydrate, uronic acid, protein and starch contents of polysaccharides were determined by a spectrophotometric method. The molecular weight distributions of polysaccharides were determined by gel filtration chromatography. Their monosaccharide composition analysis was performed by the method of 1-phenyl-3-methyl-5-pyrazolone (PMP) pre-column derivatization and RP-HPLC analysis. HRWP, LBWP, LRWP and NTWP (50, 100 and 200 mg/kg) were orally administrated to mice once daily for 15 days, respectively. Anti-fatigue activity was assessed using the forced swim test (FST), and serum biochemical parameters were determined by an autoanalyzer and commercially available kits; the body and organs were also weighted.

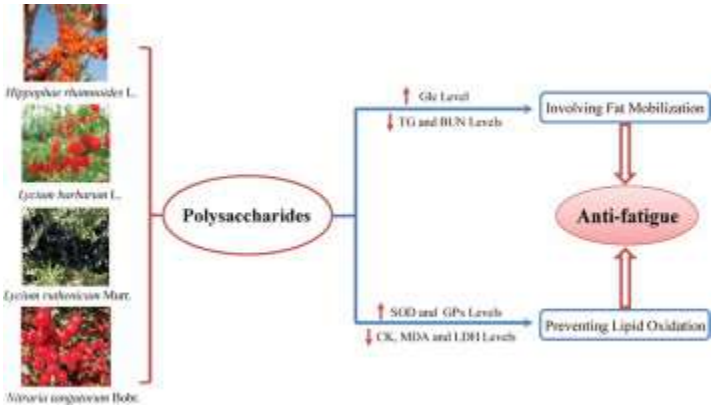
### Result

LBWP, LRWP and NTWP were mainly composed of glucans and some RG-I pectins, and HRWP was mainly composed of HG-type pectin and some glucans. All the four polysaccharides decreased immobility in the FST, and the effects of LBWP and NTWP were demonstrated in lower doses compared with HRWP and LRWP. There was no significant difference in liver and heart indices between non-treated and polysaccharide-treated mice, but the spleen indices were increased in LBWP and NTWP (200 mg/kg) group. Moreover, the FST-induced reduction in glucose (Glc), superoxide dismutase (SOD) and glutathione peroxidase (GPx) and increase in creatine phosphokinase (CK), lactic dehydrogenase (LDH), blood urea nitrogen (BUN), triglyceride (TG) and malondialdehyde (MDA) levels, all indicators of fatigue, were inhibited by HRWP, LBWP, LRWP and NTWP to a certain extent while the effects of LBWP and NTWP were much better than that of HRWP and LRWP at the same dosage.

### Conclusion

Water-soluble polysaccharides HRWP, LBWP, LRWP and NTWP, from the fruits of four Tibetan plateau indigenous berry plants, significantly exhibited anti-fatigue activities for the first time, through triglyceride (TG) (or fat) mobilization during exercise and protecting corpuscular membrane by prevention of lipid oxidation via modifying several enzyme activities. Moreover, it is demonstrated that LBWP and NTWP are more potent than HRWP and LRWP, which were proposed to be applied in functional foods for anti-fatigue and antioxidant potential.

## Graphical abstract



# Protective Effect of Lycium Barbarum Polysaccharides on Oxidative Damage in Skeletal Muscle of Exhaustive Exercise Rats

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## Abstract

The aim of this study was to determine the modulatory effect of Lycium barbarum polysaccharides (LBP) on the oxidative stress induced by an exhaustive exercise. 32 male Wistar rats were taken in the study. The experiment was a 30-day exhaustive exercise program. We determined the lipid peroxidation, glycogen levels, and anti-oxidant enzyme activities in skeletal muscle. The results demonstrated that L. barbarum polysaccharides administration significantly increases glycogen level and anti-oxidant enzyme activities, and decreased malondialdehyde (MDA) level and creatine kinase activities. In conclusion, L. barbarum polysaccharides administration can significantly decrease the oxidative stress induced by the exhaustive exercise.

# [Isolation and Purification of Lycium Barbarum Polysaccharides and Its Antifatigue Effect]

[Article in Chinese]
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## Abstract

A purified component of lycium barbarum polysaccharide (LBP-X) was isolated from lycium barbarum L. by DEAE ion-exchange cellulose and sephacryl gel chromatography. LBP-X was tested on five different doses (5, 10, 20, 50 and 100 mg.kg-1.d-1) in mice. The results showed that LBP-X induced a remarkable adaptability to exercise load, enhanced resistance and accelerated elimination of fatigue. LBP-X could enhance the storage of muscle and liver glycogen, increase the activity of LDH before and after swimming, decrease the increase of blood urea nitrogen (BUN) after strenuous exercise, and accelerate the clearance of BUN after exercise. The dosage of LBP-X 10 mg.kg-1.d-1 was the best amount among the five tested doses.

# Berry Fruits: Compositional Elements, Biochemical Activities, and the Impact

# of Their Intake on Human Health, Performance, and Disease

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## Abstract

An overwhelming body of research has now firmly established that the dietary intake of berry fruits has a positive and profound impact on human health, performance, and disease. Berry fruits, which are commercially cultivated and commonly consumed in fresh and processed forms in North America, include blackberry ( Rubus spp.), black raspberry ( Rubus occidentalis), blueberry ( Vaccinium corymbosum), cranberry (i.e., the American cranberry, Vaccinium macrocarpon, distinct from the European cranberry, V. oxycoccus), red raspberry ( Rubus idaeus) and strawberry ( Fragaria x ananassa). Other berry fruits, which are lesser known but consumed in the traditional diets of North American tribal communities, include chokecherry ( Prunus virginiana), highbush cranberry ( Viburnum trilobum), serviceberry ( Amelanchier alnifolia), and silver buffaloberry ( Shepherdia argentea). In addition, berry fruits such as arctic bramble ( Rubus articus), bilberries ( Vaccinuim myrtillus; also known as bog whortleberries), black currant ( Ribes nigrum), boysenberries ( Rubus spp.), cloudberries ( Rubus chamaemorus), crowberries ( Empetrum nigrum, E. hermaphroditum), elderberries ( Sambucus spp.), gooseberry ( Ribes uva-crispa), lingonberries ( Vaccinium vitis-idaea), loganberry ( Rubus loganobaccus), marionberries ( Rubus spp.), Rowan berries ( Sorbus spp.), and sea buckthorn ( Hippophae rhamnoides), are also popularly consumed in other parts of the world. Recently, there has also been a surge in the consumption of exotic "berry-type" fruits such as the pomegranate ( Punica granatum), goji berries ( Lycium barbarum; also known as wolfberry), mangosteen ( Garcinia mangostana), the Brazilian açaí berry ( Euterpe oleraceae), and the Chilean maqui berry ( Aristotelia chilensis). Given the wide consumption of berry fruits and their potential impact on human health and disease, conferences and symposia that target the latest scientific research (and, of equal importance, the dissemination of this information to the general public), on the chemistry and biological and physiological functions of these "superfoods" are necessary.

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*Article*

# *Lycium barbarum* Polysaccharides Reduce Exercise-Induced Oxidative Stress

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**Abstract:** The purpose of the present study was to investigate the effects of *Lycium barbarum* polysaccharides (LBP) on exercise-induced oxidative stress in rats. Rats were divided into four groups, *i.e.*, one control group and three LBP treated groups. The animals received an oral administration of physiological saline or LBP (100, 200 and 400 mg/kg body weight) for 28 days. On the day of the exercise test, rats were required to run to exhaustion on the treadmill. Body weight, endurance time, malondialdehyde (MDA), super oxide dismutase (SOD) and glutathione peroxidase (GPX) level of rats were measured. The results showed that the body weight of rats in LBP treated groups were not significantly different from that in the normal control group before and after the experiment ($P > 0.05$). After exhaustive exercise, the mean endurance time of treadmill running to exhaustion of rats in LBP treated groups were significantly prolonged compared with that in the normal control group. MDA levels of rats in LBP treated groups were significantly decreased compared with that in the normal control group ($P < 0.05$). SOD and GPX levels of rats in LBP treated groups were significantly increased compared with that in the normal control group ($P < 0.05$). Together, these results indicate that LBP was effective in preventing oxidative stress after exhaustive exercise.

**Keywords:** *Lycium barbarum* polysaccharides; exercise; oxidative

## 1. Introduction

*Lycium barbarum* belongs to the plant family Solanaceae. Red-colored fruits of *Lycium barbarum*, also called *Fructus lycii* or *Gouqizi*, have been used as a traditional Chinese herbal medicine for thousands of years [1]. In traditional Chinese medicine literature, it has been known for balancing "Yin" and "Yang" in the body, nourishing the liver and kidney and improving visual acuity [2,3]. *Lycium barbarum* fruits have a large variety of biological activities and pharmacological functions and play an important role in preventing and treating various chronic diseases, such as diabetes, hyperlipidemia, cancer, hepatitis, hypo-immunity function, thrombosis, and male infertility [4–7]. In fact, in 1983 the Ministry of the Public Health of China approved *Lycium barbarum* fruits to be marketed as a botanical medicine. Various chemical constituents are found in *Lycium barbarum* fruits. The polysaccharides isolated from the aqueous extracts of *Lycium barbarum* have been identified as one of the active ingredients responsible for the biological activities [7,8]. Previous studies have shown that *Lycium barbarum* polysaccharides (LBP) can enhance exercise endurance capacity, reduce fatigue and exhibit antioxidant activity *in vitro* and *in vivo* [9–13].

Regular physical exercise has many health benefits including a lowered threat of all-cause mortality along with a reduced risk of cardiovascular disease, cancer, and diabetes [14–16]. However, strenuous physical exercise with dramatically increased oxygen uptake is associated with the generation of free radicals and reactive oxygen species (ROS), such as superoxide and hydrogen peroxide, which might cause lipid peroxidation of polyunsaturated fatty acids in membranes, DNA damage, and decreases antioxidant levels in target tissues and blood [17–19]. Oxidative stress can be defined as an imbalance between oxidative reactions and antioxidant capacity that results directly or indirectly in cellular damage [20]. During the past three decades, our knowledge about the biological implications of exercise-induced oxidative stress has expanded rapidly.

Antioxidants are substances that help reduce the severity of oxidative stress either by forming a less active radical or by quenching the reaction. The literature suggests that dietary antioxidants may prevent muscle damage because they are able to detoxify some peroxides by scavenging ROS produced during exercise [21–25]. *Lycium barbarum* polysaccharides (LBP), due to their antioxidant properties, may be applicable in the treatment of disorders in which oxidative stress is involved, including exercise-induced oxidative stress. Therefore, the purpose of this study was to investigate the effects of LBP on exercise-induced oxidation in male rats.

## 2. Results and Discussion

### *2.1. Effects of LBP on Body Weight and Endurance Time of Rats*

As shown in Table 1, the body weight of rats in LBP treated groups (low-dose LBP treated (LT), middle-dose LBP treated (MT) and high-dose LBP treated (HT)) were not significantly different from those in the normal control group (NC) before and after the experiment ($P > 0.05$), which means the LBP had no effect on body weight. The mean endurance time of treadmill running to exhaustion of rats in LBP treated groups (LT, MT and HT) were significantly prolonged compared to that in the normal control group (NC) ($P < 0.05$), which was 1.38, 1.45 and 1.55 times that in the NC group, respectively.

The results suggested that different doses of LBP might significantly prolong the endurance time, which suggests that LBP might elevate the exercise tolerance of rats.

**Table 1.** Effects of *Lycium barbarum* polysaccharides **(**LBP) on body weight and endurance time of rats (mean ± SD, n = 12).

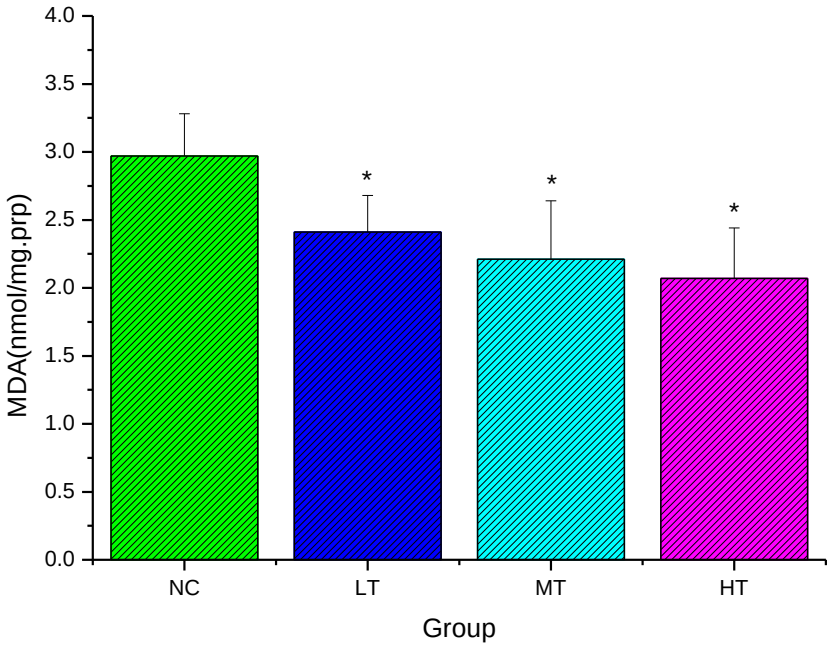
| Group | Body weight (g) | | Endurance time (min) |
|---|---|---|---|
| | Before experiment | After experiment | |
| NC | 284.61 ± 28.46 | 434.54 ± 31.28 | 61.21 ± 4.22 |
| LT | 289.49 ± 21.37 | 427.39 ± 27.23 | 84.37 ± 6.28* |
| MT | 292.34 ± 24.61 | 441.06 ± 22.84 | 88.94 ± 5.76* |
| HT | 287.59 ± 30.25 | 429.17 ± 25.62 | 94.79 ± 5.94* |

*$p < 0.05$ as compared with the normal control group (NC).

*2.2. Effects of LBP on Malondialdehyde (MDA) Level of Rats after Exhaustive Exercise*

Malondialdehyde (MDA) has been the most widely used parameter for evaluating oxidative damage to lipids, although it is known that oxidative damage to amino acids, proteins and DNA also causes release of MDA. Most studies show that endurance exercise causes an increase in MDA [26–28]. As shown in Figure 1, after exhaustive exercise, MDA levels of rats in the LBP treated groups (LT, MT and HT) were significantly decreased compared with those in the normal control group (NC) ($P < 0.05$). The results suggested that different doses of LBP could reduce lipid per-oxidation during exercise.

**Figure 1.** Effects of LBP on malondialdehyde (MDA) level of rats after exhaustive exercise (mean ± SD, n = 12). *$p < 0.05$ as compared with the normal control group (NC).



### *2.3. Effects of LBP on Super Oxide Dismutase (SOD) and Glutathione Peroxidase (GPX) Level of Rats after Exhaustive Exercise*

Antioxidant enzymes, which provide the primary defense against ROS generated during exercise, may be activated selectively during an acute bout of strenuous exercise depending on the oxidative stress imposed on the specific tissues as well as the intrinsic antioxidant defense capacity [16,29,30]. Superoxide dismutase reduces superoxide to hydrogen peroxide; and glutathione peroxidase reduces hydrogen peroxide from the SOD reaction to water. In addition, glutathione peroxidase can reduce lipid peroxides directly [31,32]. As shown in Table 2, after exhaustive exercise, SOD and GPX levels of rats in the LBP treated groups (LT, MT and HT) were significantly increased compared with those in the normal control group (NC) ($P < 0.05$). The results indicate that different doses of LBP were able to up-regulate antioxidant enzyme activities to protect against oxidative stress induced by acute exercise. This is probably due to the antioxidant activity of LBP *per se*.

**Table 2.** Effects of LBP on super oxide dismutase (SOD) and glutathione peroxidase (GPX) levels of rats after exhaustive exercise (mean ± SD, n = 12).

| Group | SOD (U/mg pro) | GPX(U/mg pro) |
|---|---|---|
| NC | 101.48 ± 10.28 | 4.74 ± 1.25 |
| LT | 131.36 ± 9.41* | 7.23 ± 0.96* |
| MT | 148.69 ± 11.23* | 10.37 ± 1.14* |
| HT | 157.84 ± 12.65* | 14.29 ± 1.29* |

*$p < 0.05$ as compared with the normal control group (NC).

## 3. Experimental Section

### *3.1. Chemicals*

Reagent kits for the determination of malondialdehyde (MDA), super oxide dismutase (SOD) and glutathione peroxidase (GPX) were purchased from Jiancheng Biotechnology Co. (Nanjing, China). All other reagents were purchased from either Sigma Chemical Co. (St. Louis, U.S.) or Sinopharm Chemical Reagent Beijing Co., Ltd (Beijing, China).

### *3.2. Plant Materials*

The dried *Lycium barbarum* fruits were purchased from Hangzhou city herb market (Zhejiang, China). The plants were identified by Professor Li in the Institute of Zhejiang Institute of Botany, China. A voucher specimen (ZJB-67581) is deposited in the Herbarium of the Zhejiang Institute of Botany.

### *3.3. Preparation of Lycium Barbarum Polysaccharides*

*Lycium barbarum* polysaccharides were prepared as described previously [1,33,34]. In brief, 100 g of dried fruit were ground to fine powder and put in 1.5 L of boiling water and decocted for 2 h by a traditional method for Chinese medicinal herbs. The decoction was left to cool at room temperature,

filtered and then freeze-dried to obtain crude polysaccharides. The dried crude polysaccharides were refluxed three times to remove lipids with 150 mL of chloroform:methanol solvent (2:1) (v/v). After filtering, the residue was air-dried. The resulting product was extracted three times in 300 mL of hot water (90 ℃) and then filtered. The combined filtrate was precipitated using 150 mL of 95% ethanol, 100% ethanol and acetone, respectively. After filtering and centrifugation, the precipitate was collected and vacuum-dried, giving the desired *Lycium barbarum* polysaccharides (LBP). The content of LBP was measured by phenol sulfuric method [35]. Results showed that the content of the polysaccharides in the extract may reach 95.18%.

### *3.4. Animals and Treatments*

Eight-week-old male Sprague-Dawley rats, weighing 280 to 300 g, were purchased from Hangzhou animal husbandry center (Zhejiang, China). Rats were maintained on a 12-hour light/dark cycle (lights on 07:00–19:00 hours) in a constant temperature (21–23 ℃) and 55 ± 10% relative humidity colony room, with free access to food and water. The approval for this experiment was obtained from the Institutional Animal Ethics Committee of Zhejiang University of Technology (Zhejiang, China). After an adaptation period of a week, 48 rats were randomly divided into four groups, *i.e.*, one control group and three LBP treated groups, of 12 each (Table 3). The volume of administration was 1 mL and the treatments lasted for 28 days. Before the formal experiments, some preliminary experiments were done, and the doses of LBP were determined to be 50 to 600 mg/kg according to relevant literature [36–38]. The results of the preliminary experiments showed that doses of 100 to 400 mg/kg were suitable and effective, with no toxicity in mice. Thus, in this study, the doses of LBP of 100 mg/kg, 200 mg/kg and 400 mg/kg b.w were chosen.

**Table 3.** Grouping of animals.

| **Group** | **Number** | **Administration of animals** |
|---|---|---|
| Normal control (NC) | 12 | Rats were treated orally with physiological saline every day. |
| Low-dose LBP treated (LT) | 12 | Rats were treated orally with 100 mg/ kg b.w. LBP every day. |
| Middle-dose LBP treated (MT) | 12 | Rats were treated orally with 200 mg/ kg b.w. LBP every day. |
| High-dose LBP treated (HT) | 12 | Rats were treated orally with 400 mg/ kg b.w. LBP every day. |

### *3.5. Exercise Protocol*

Rats were introduced to treadmill running with 15–20 min exercise bouts at 15–30 m/min for a week to accustom them to running. On the day of the exercise test (the last day of treatment ), rats were required to run to exhaustion on the treadmill at a final speed of 30 m/min, 10% gradient and approximately 70–75% $VO_2$max (Liu *et al.*, 2005). The point of exhaustion was determined when the rat was unable to right itself when placed on its back. The treadmill was provided from Zhishuduobao Biological Technology Company (DB030l device; Beijing, china).

### *3.6. Sample Preparation*

All animals were anesthetized with ethyl ether and sacrificed immediately after the exhaustive exercise. Hind-limb skeletal muscle was extracted and frozen in liquid nitrogen for storage at −80 ℃ until further analysis.

### *3.7. Analytical Oxidative Stress-Associated Parameters*

The tissues were homogenized in ice-cold buffer (0.25 M sucrose, 10 mM Tris-HCl, and 0.25 mM phenylmethylsulfonyl fluoride; pH 7.4), and a portion of the homogenate was measured immediately for malondialdehyde (MDA) using a commercial diagnostic kit. Another portion of the homogenate was centrifuged at 10,000 × g for 20 min at 4 ℃; super oxide dismutase (SOD) and glutathione peroxidase (GPX) activities in the supernatant were measured using commercial diagnostic kits.

### *3.8. Statistical Analysis*

All values are expressed as mean ± standard deviation. Statistical comparisons were made by one-way ANOVA and correlation analysis was performed by Pearson product moment using SPSS version 13.0 (SPSS Inc., Chicago, IL, U.S.). Statistical significance was defined as $P < 0.05$.

## 4. Conclusions

The present results suggest that *Lycium barbarum* polysaccharides (LBP) could elevate the exercise tolerance, reduce lipid per-oxidation and up-regulate antioxidant enzyme activity during exercise. This indicates that LBP is effective in preventing oxidative stress after exhaustive exercise.

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# Regulatory effects of the fruit extract of *Lycium chinense* and its active compound, betaine, on muscle differentiation and mitochondrial biogenesis in C2C12 cells

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ABSTRACT

Our study was conducted to investigate the effects of the fruits of *Lycium chinense* Mill. (Lycii Fructus, LF) and its bioactive compound, betaine, on muscle differentiation and mitochondrial biogenesis in C2C12 cells. LF extract and betaine was analyzed by high-performance liquid chromatography (HPLC). The expression of myosin heavy chain (MyHC) and peroxisome proliferator-activated receptor gamma coactivator1-alpha (PGC-1α), sirtuin-1(Sirt-1), nuclear respiratory factor-1 (NRF-1), transcription factor A, mitochondrial (TFAM) and the phosphorylation of AMP-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC), were determined in cellular or mitochondrial levels by quantitative polymerase chain reaction (qPCR) or Western blot, respectively. The glucose levels and total ATP contents were measured by the glucose consumption in a culture medium, cellular glucose uptake and ATP assays. LF extract at 4 mg/ml and betaine at 2 and 5 mM significantly increased the expression of MyHC in C2C12 myotubes, compared with non-treated cells. LF extract and betaine significantly increased the expression of PGC-1α, Sirt-1, NRF-1 and TFAM mRNA and protein in the myotubes, as well as phosphorylation of AMPK and ACC. Furthermore, LF extract and betaine significantly increased the mitochondrial protein contents, as the TFAM and NRF-1 expressions were increased. LF extract and betaine also significantly increased the glucose uptake and ATP contents in the myotubes. The LF extract contained 3.18% betaine was quantitated by HPLC. LF extract and betaine enhanced muscle differentiation and energy metabolism through the up-regulation of mitochondrial biogenesis-regulating factors, suggesting that LF extract and betaine can help to prevent the dysfunction of skeletal muscle.

## 1. Introduction

Skeletal muscle is largely responsible for regulating carbohydrate metabolism and achieving energy balance in the human body under normal feeding conditions [1]. Mitochondria are essential eukaryotic organelles, whose most important function is to provide cellular energy, in the form of ATP [2], and play a fundamental role in biological processes, such as amino acid metabolism and ion homeostasis [3]. In muscle, to transition from the glycolytic state of myoblasts, the mitochondria must acquire a dramatically different repertoire in order to support the metabolic demands of the differentiated myotubes [4]. Therefore, the up-regulation of the mitochondrial function promotes the myoblast differentiation process and muscle functions.

Nowadays, various products of the fruits of *Lycium chinense* Mill. Or *L. barvarum* L. (Lycii Fructus; LF) are used as food or therapeutic materials and have become increasingly popular all over the world. In traditional Chinese medicine (TCM) and Korean medicine (TKM), LF is applied in *yin* deficiency syndromes of the liver and kidney, which involvedizziness, aching and limpness in the loins and knees, spermatorrhea, deafness, loosened teeth, early graying of hair, insomnia and dream-disturbed sleep due to an insufficiency of essence and blood, tidal fever, and night sweating. In pharmacological studies, this plant is reported to have various effects, such as anti-oxidation [5], anticancer [6], anti-diabetes [7], neuro-protection [8], hepatoprotection [9] and

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immunomodulation [10] LF contains various constituents, such as polysaccharides, amino acids, fatty acids [11], carotenoids, phenolic acids and flavonoids [12]. Betaine, a trimethyl derivative of the amino acid, glycine, is one of the main bioactive compounds in LF extract [13] and was reported to have ergogenic effects in a clinical study [14], as well as anti-inflammatory [15], anti-fatigue [16], and anti-aging [17] effects.

In this study, we evaluated the beneficial effects of LF extract and betaine on muscle differentiation and energy metabolism through the regulation of mitochondrial biogenesis regulators in C2C12mouse skeletal muscle cells.

## 2. Materials and methods

### 2.1. Materials

Betaine and metformin were purchased from Sigma-Aldrich (St. Louis, MO, USA). DMEM and penicillin/streptomycin solution were acquired from Corning (Manassas, NY, USA). Fetal bovine serum (FBS) and horse serum (HS) were obtained from Merck Millipore (Temecula, CA, USA). An ATP colorimetric assay kit was purchased from BioVision Inc. (Milpitas, CA, USA). Anti-Sirt-1, anti-TFAM, anti-NRF-1, anti-AMPK, anti-phospho-AMPK, anti-total-AMPK, anti-phospho-ACC, and anti-total-ACC antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-MyHC and anti-GLUT4 antibodies were acquired from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-PGC-1α antibody and radio-immunoprecipitation assay (RIPA) buffer were obtained from Thermo Fisher Scientific (Waltham, MA, USA).

### 2.2. Preparation of LF extract

Dried fruits of L.chinense (Lycii Fructus; LF) were purchased from herbal company (Kwangmyungdang, Ulsan, Korea) and identified by Professor Y.-K. Park, a medical botanist in herbology at the College of Korean Medicine, Dongguk University (DUCOM), Korea. A voucher specimen was deposited at the herbarium of DUCOM (2017-LF-W). Briefly, LF (200 g) was extracted by boiling it in 2 L of water for 3 h, filtering it through Whatman grade1 filter paper (Sigma-Aldrich), concentrating it under a vacuum rotary evaporator (EyelaCo. Ltd., Tokyo, Japan) at 60 °C, and then lyophilizing it in a freeze-dryer (IlShin BioBase Co., Yangju, Korea) at −80 °C under 5mTorr. LF extract (yield: 40.45%) was stored at 4 °C, dissolved in1 × PBS, and filtered through a syringe filter (0.45 μm, Corning), before being used in in vitro experiments.

### 2.3. Cell culture and drug treatments

The 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%penicillin/streptomycin solution, in a humidified atmosphere of 95%air and 5%$CO_2$ at 37 °C. At 85–95% confluence, myoblasts were induced to differentiate in DMEM using 2%horse serum (HS) once every day for an additional 5days. The C2C12 myotubes were then treated with or without different concentrations of LF extract (2 and 4 mg/ml) or betaine (2 and 5 mM) for 24 h. Metformin (2.5 mM) was used as a positive control drug. Betaine and metformin were dissolved in 1 × PBS (pH 7.4).

### 2.4. .Western blot analysis

After cells were lysed in ice-cold RIPA buffer, containing a phosphatase inhibitor cocktail (Quartett, Berlin, Germany), lysates were centrifuged at 12,000 × *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, 30 μg of protein was separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane. The membrane was then blocked with 5% skim milk for 1 h at room temperature, after which time, it was immunoblotted with primary antibodies against MyHC, PGC-1α, NRF-1, TFAM, Sirt-1, 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, and 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 ChemiDoc MP Imaging System (BioRad) and quantified by densitometry using the ImageJ programming software (Image J 2, NIH, Bethesda, MD, USA).

### 2.5. Quantitative PCR assay

Total RNA was isolated from cells by NucleoZOL reagent (Macherey-Nagel GmbH&Co, KG, Neumann-Neander, Germany), according to the manufacturer's instructions. The RNA concentration was then quantified using a Nano Drop ND-1000 spectrophotometer (Nano Drop Technologies, Inc., Wilmington, DE, USA). Next, cDNA was generated from 1 μg of total RNA using a ReverTra qPCR RT master mix kit (Toyobo, Osaka, Japan), following the manufacturer's instructions. PCR was performed using a SYBR Green kit (Agilent Technologies, West Cedar Creek, TX, USA) and primers specific to the target genes (Table 1). Gene expression was evaluated by the ΔΔCT method, using GAPDH as a housekeeping gene.

### 2.6. Immunocytochemistry

The C2C12 myoblast were seeded on Thermanox plastic cover slips (Nunc™, Thermo Fisher Scientific) and differentiated using a common culture method for 5days. Determination of MyHC performed after 24 h drugs treatment. For determination of GLUT4 translocation, the differentiated C2C12 myotubes were serum-starved for 4 h and then treated with drugs for 6 h. After treatment samples on cover slips were washed with 1 × PBS and fixed with 4%paraformaldehyde for 10 min, after which time, they were permeabilized with 0.1%Triton X-100 (Sigma-Aldrich) for 20 min. After being washed with 1 × PBS, the cover slips were blocked with 5% bovine serum albumin (BSA) for 30 min at RT and then incubated with anti-MyHC or anti-GLUT4 antibody overnight at 4 °C. The cover slips were subsequently labeled with Alexa Fluor 488-conjugated goat anti-rabbit antibody for 1 h at RT, then counterstained with DAPI for 5 min. Finally, the expression of MyHC was observed using a fluorescence microscope (Leica DM2500, Leica microsystems, Wetzlar, Germany).

**Table 1**
Primer sequences of the target genes for qPCR.

| Primers | | Accession No. | Sequence (5′→3′) |
|---|---|---|---|
| **MyHC** | Forward | NM 030679.1 | AGT CCC AGG TCA ACA AGC TG |
| | Reverse | NM 030679.1 | TTC CAC CTA AAG GGC TGT TG |
| **PGC-1α** | Forward | XM 006503779.3 | CAC CAA ACC CAC AGA AAA CAG |
| | Reverse | XM 006503779.3 | GGG TCA GAG GAA GAG ATA AAG TTG |
| **NRF-1** | Forward | XM 017321445.1 | AGG GCG GTG AAA TGA CCA TC |
| | Reverse | XM 017321445.1 | CGG CAG CTT CAC TGT TGA GG |
| **TFAM** | Forward | XM 017313918.1 | GGG TAT GGA GAA GGA GGC CC |
| | Reverse | XM 017313918.1 | TCC CTG AGC CGA ATC ATC CT |
| **Sirt-1** | Forward | NM 001159589.2 | GAT CCT TCA GTG TCA TGG TT |
| | Reverse | NM 001159589.2 | GAA GAC AAT CTC TGG CTT CA |
| **GAPDH** | Forward | XM_017321385.1 | CAG CCT CGT CCC GTA GAC A |
| | Reverse | XM_017321385.1 | CGC TCC TGG AAG ATG GTG AT |

### 2.7. Glucose assay

Glucose consumption was determined in culture media using a glucose assay kit (Sigma-Aldrich). Briefly, the cell culture medium was harvested and diluted with deionized water, after which time, 50 µl of the 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 $H_2SO_4$ and then measured at 540 nm in a microtiter reader (UVM340, Asys Hitech Gmbh, Eugendorf, Austria). The glucose consumption in each sample was calculated using a calculation formula from a standard curve.

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, the myotubes were treated with or without LF extract and betaine at different concentrations in a 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 h. 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 at 485/650 [excitation/emissionusing a Glomax multi detection system (Promega Biosystems, Sunnyvale, CA, USA)].

### 2.8. ATP assay

The 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 cell lysate was mixed with 50 µl of the reaction mix, containing the ATP probe, converter, and developer in a 96-well plate. The mixture was subsequently incubated at room temperature for 30 min, at which time, 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. Mitochondria isolation

Mitochondria were isolated from the cells using a mitochondria isolation kit for mammalian cells (Thermo Fisher Scientifc, Rockford, IL, USA), according to the manufacturer's protocol. Briefly, C2C12 myotube cells were harvested and washed twice in ice-cold 1 × PBS. The cells were lysed with 800 µl isolation reagent A at the vortex medium speed for 5 s, after which time,10 µl of mitochondria isolation reagent B was added at the vortex maximum speed for 5 s. Later, lysates were incubated on ice for 5 min and vortexed at maximum speed every minute. Then, 800 µl mitochondria isolation reagent C was added, supplemented with a proteinase inhibitor (Quartett, Berlin, Germany), and the mixture was centrifuged at 700 × *g* for 10 min at 4 °C. Next, further supernatant was transferred and centrifuged at 12,000 × *g* for 15 min at 4 °C, and the supernatant was discarded (cytosol fraction). The pellet containing the isolated mitochondria was suspended in 500 µl of mitochondria isolation reagent C and collected the final mitochondrial fraction by centrifugation at 12,000 × *g* for 5 min at 4 °C. The concentration of mitochondrial protein was determined using the Bradford assay (BioRad,Hercules, CA, USA).

### 2.10. HPLC analysis

To identify betaine in LF extract, HPLC was conducted using an Agilent1260 infinity II quaternary system, equipped with a G7129A vial sampler and Evaporative light scattering detectors (Agilent, Waldbronn, Germany) and a Tnature C18 column (4.6 × 250 mm, 5 µm). Chromatographic separation was performed using an isocratic solvent system, consisting of acetonitrile (A) (HPLC grade, Merck, Darmstadt, Germany) and water (B) (HPLC grade, Merck), for 0–10 min (A:B = 1:99). The analysis was carried out using ELSD detection at a flow rate of 1.0 ml/min. The ELSD parameters for the spray chamber, drift tube temperature and gas pressure were optimized at 30 °C, 80 °Cand 60 psi, respectively.

### 2.11. Statistical analysis

The data are presented as the means ± standard errors of means (SEMs) of three independent experiments. The differences between the groups were identified by the Student's *t*-test using the GraphPad Prism program (ver.5.0, GraphPad Software, La Jalla, CA, USA), and the *p*-values $< 0.05$ were considered statistically significant.

## 3. Results

### 3.1. Effects of LF extract and betaine on myotubes differentiation

To investigate the effects of LF extract and betaine on myoblasts differentiation in myotubes, we observed the expression of MyHC mRNA and protein as a differentiation marker using qPCR and Western blot, respectively. As a result, treatment with LF extract ($p < 0.05$ for 4mg/ml) and betaine ($p < 0.01$ for 2mM and $p < 0.001$ for 5mM) significantly increased the expression of MyHC protein (Fig.1A) and mRNA (Fig.1B) in C2C12 myotubes, compared with non-treated cells. In immunocytochemistry (Fig.1C), the treatment of LF extract and betaine increased the expression of MyHC, which was shown to have an elongated and widened cylinder shape, with multiple nuclei in, and this was dose-dependent. Metformin-treated cells were shown in the increase of the MyHC expression, but they were fewer than in the LF extract and betaine treatment. These results indicate that LF extract and betaine can help to stimulate muscle differentiation.

C2C12 myoblasts were differentiated using DMEM containing 2% HS for 5 days, then treated with or without LF extract (2 and 4 mg/ml) or betaine (2 and 5 mM) for 24 h. Metformin (2.5 mM) was used as a positive control. The expression of MyHC protein (A) and mRNA (B) was determined by RT-PCR and Western blot, respectively. β-Actin and GAPDH were used as internal controls. All data were presented as the means ± SEM of three independent experiments. N, normal cell; LF, LF extract; B, betaine; and M, metformin. *p < 0.05, **p < 0.01and ***p < 0.001 vs. non-treated cells; (C) the myotubes were stained with anti-MyHC antibody and DAPI and then observed by fluorescence microscopy (original magnification = 200 ×). Green, MyHC-positive cells; and blue, DAPI-positive nuclei.

### 3.2. Effects of LF extract and betaine on the expression of mitochondrial biogenesis-regulating factors in myotubes

To investigate the effects of LF extract and betaine on biogenesis in muscle cells, we measured the expression of mitochondrial biogenesis-regulating factors, PGC-1α, NRF-1, TFAM, and Sirt-1 mRNAand protein in C2C12 myotubes by qPCR and Western blot. As a result, treatment with LF extract at high concentration and betaine significantly increased the expression of PGC-1α ($p < 0.01$ for LF 4 mg and betaine 5 mM, $p < 0.05$ for betaine 2 mM, Fig.2A), NRF-1 ($p < 0.05$, Fig.2B),TFAM ($p < 0.01$ for LF 4mg and betaine 2mM, $p < 0.001$ for betaine 5 mM, Fig.2C) and Sirt-1 ($p < 0.05$ for LF 4 mg and $p < 0.01$ for betaine 2 mM, Fig.2D) mRNA in C2C12 myotubes. As for the protein levels, the expression of PGC-1α ($p < 0.01$, Fig.3A), NRF-1 ($p < 0.01$ for LF 4 mg and betaine 2 mM, $p < 0.001$ for betaine 5 mM, Fig.3B), TFAM ($p < 0.01$, Fig.3C) and Sirt-1 ($p < 0.05$ for LF 4 mg and $p < 0.01$ for betaine 5 mM, Fig.3D) were significantly increased in LF extract and betaine-treated myotubes. Metformin also significantly increased the expression of PGC-1α ($p < 0.001$ for protein and mRNA) and NRF-1 ($p < 0.01$ for protein), TFAM ($p < 0.05$ for protein), and Sirt-1 ($p < 0.01$ for protein) in the myotubes. These results indicate

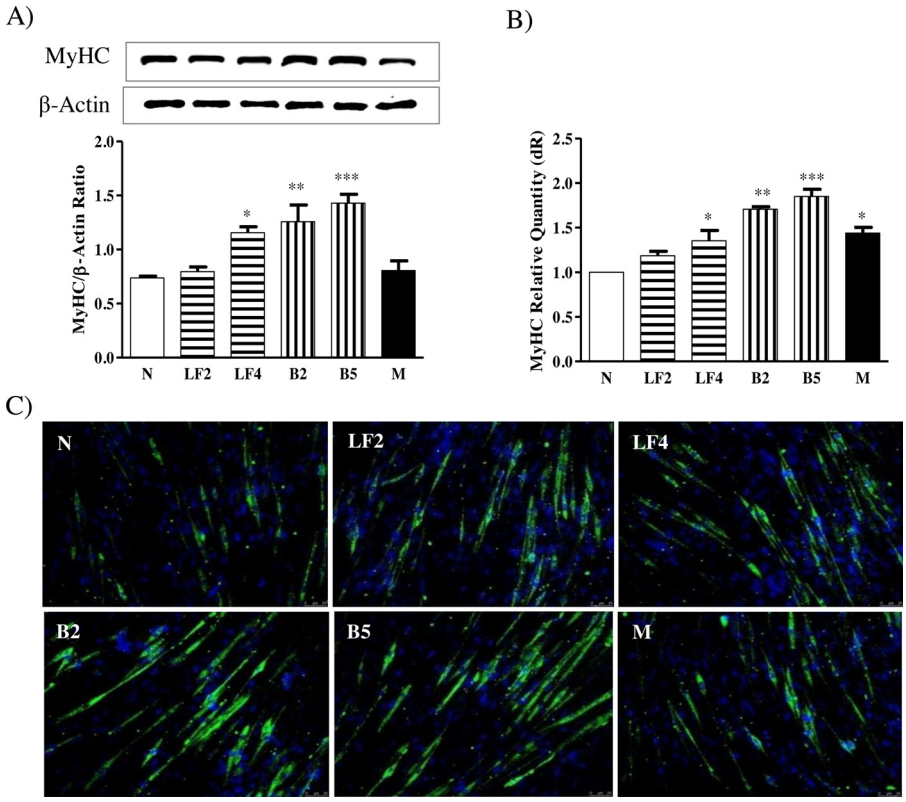


**Fig. 1.** Effect of LF extract and betaine on the expression of MyHC in C2C12 myotubes.

that LF extract and betaine improve mitochondrial biogenesis through the up-regulation of the transcription factors.

Differentiated myotubes were treated with or without LF extract (2 and 4 mg/ml) or betaine (2 and 5 mM) for 24 h, after which time, the expression of PGC-1α (A), NRF-1 (B), TFAM (C) and Sirt-1 (D) mRNA was analyzed by qPCR. Metformin (2.5 mM) was used as a positive control. GAPDH was used as an internal control. 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 ± SEM of three independent experiments. N, normal cell; LF, LF extract; B, betaine; and M, metformin. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs. non-treated cells.

Differentiated myotubes were treated with or without LF extract (2 and 4 mg/ml) or betaine (2 and 5 mM) for 24 h, and the expression of PGC-1α (A), NRF-1 (B), TFAM (C) and Sirt-1 (D) proteins was analyzed by Western blot. Metformin was used as a positive control. β-Actin was

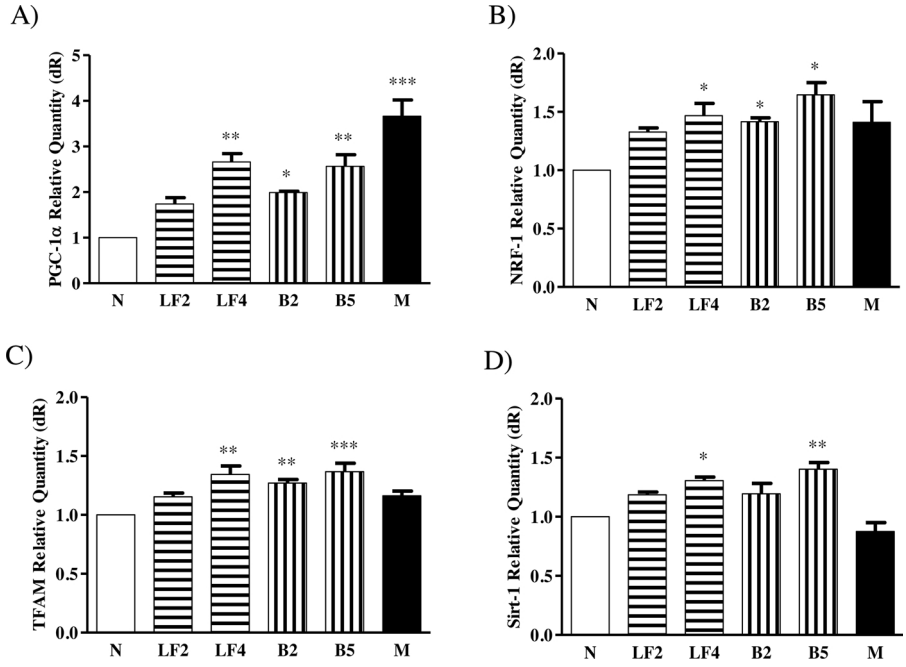


**Fig. 2.** Effect of LF extract and betaine on the mRNA expression of mitochondrial biogenesis-regulating factors in C2C12 myotubes.

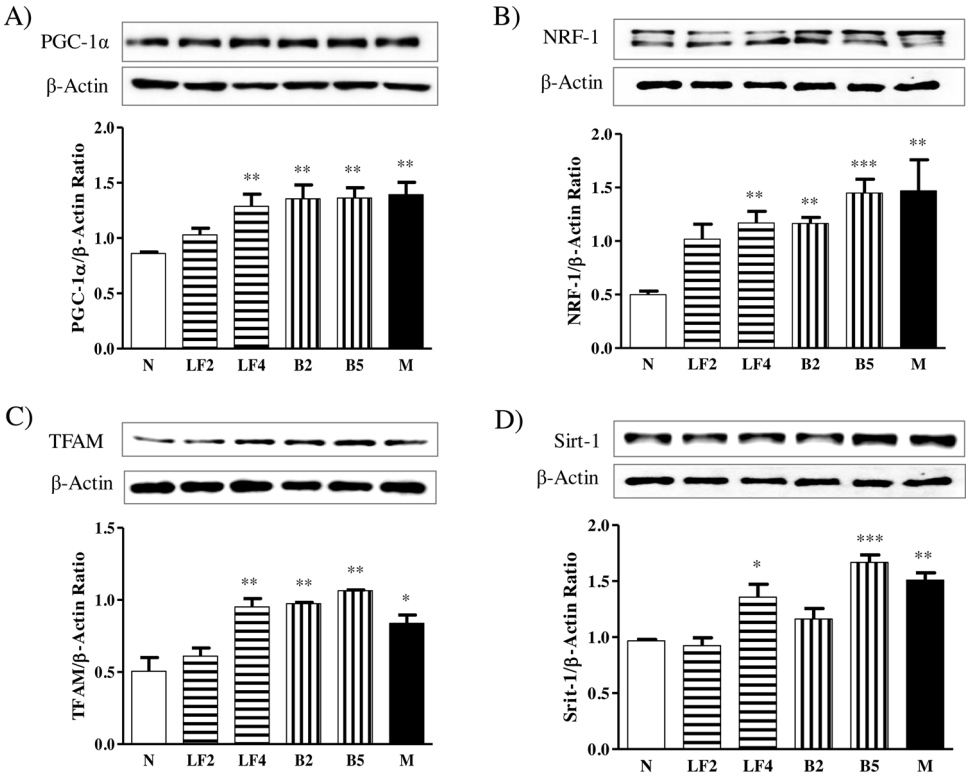


**Fig. 3.** Effect of LF extract and betaine on the protein expression of mitochondrial biogenesis-regulating factors in C2C12 myotubes.

used as an internal control. 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 ± SEM of three independent experiments. N, normal cell; LF, LF extract; B, betaine; and M, metformin. *p < 0.05, **p < 0.01 and ***p < 0.001 vs. non-treated cells.

### 3.3. Effects of LF extract and betaine on the AMPK and ACC signaling pathways in myotubes

Next, we investigated the effects of LF extract and betaine on the AMPK and ACC signaling pathway, which activates mitochondrial biogenesis in myotubes. As a result, treatment with LF extract at high concentration significantly increased phosphorylation of AMPK ($p < 0.01$, Fig.4A) and ACC ($p < 0.05$, Fig.4B), compared with non-treated cells. Betaine at high concentrations also significantly increased their expression ($p < 0.001$ for AMPK and $p < 0.05$ for ACC).In metformin-treated cells, the phosphorylation of AMPK ($p < 0.001$) and ACC ($p < 0.001$) was significantly increased, compared with non-treated cells. These results indicate that LF extract and betaine can enhance mitochondrial biogenesis in myotubes through the activation of the AMPK/ACC signaling pathway.

The phosphorylation of AMPK (A) and ACC (B) protein was investigated by Western blot. Metformin was used as a positive control. 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 ± SEM of three independent experiments. LF, LF extract; N, normal cell; LF, LF extract; B, betaine; and M, metformin. *p < 0.05, **p < 0.01 and ***p < 0.001 vs. non-treated cells.

### 3.4. Effects of LF extract and betaine on glucose uptake and energy production in myotubes

To investigate the effects of LF extract and betaine on glucose uptake in cells and energy production, we determined the expression of the GLUT-4 protein by Western blot and visualized GLUT4 translocation by immunocytochemistry, we also determined the glucose consumption in a culture medium, cellular glucose uptake and ATP contents in C2C12 myotubes. As a result, the expression of GLUT-4 protein was

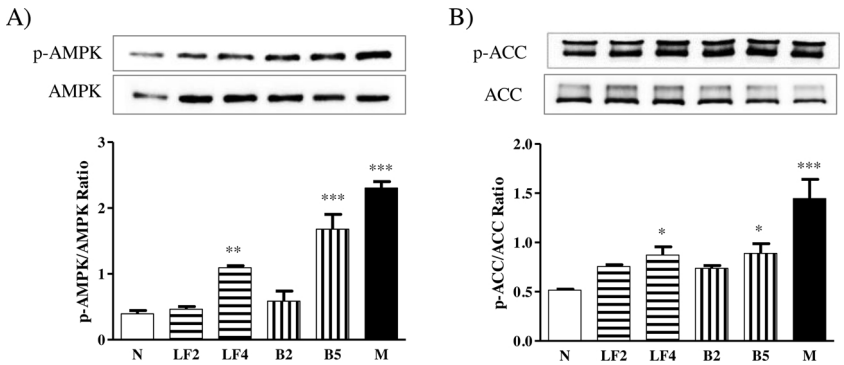


**Fig. 4.** Effect of LF extract and betaine on the phosphorylation of AMPK and ACC in C2C12 myotubes.

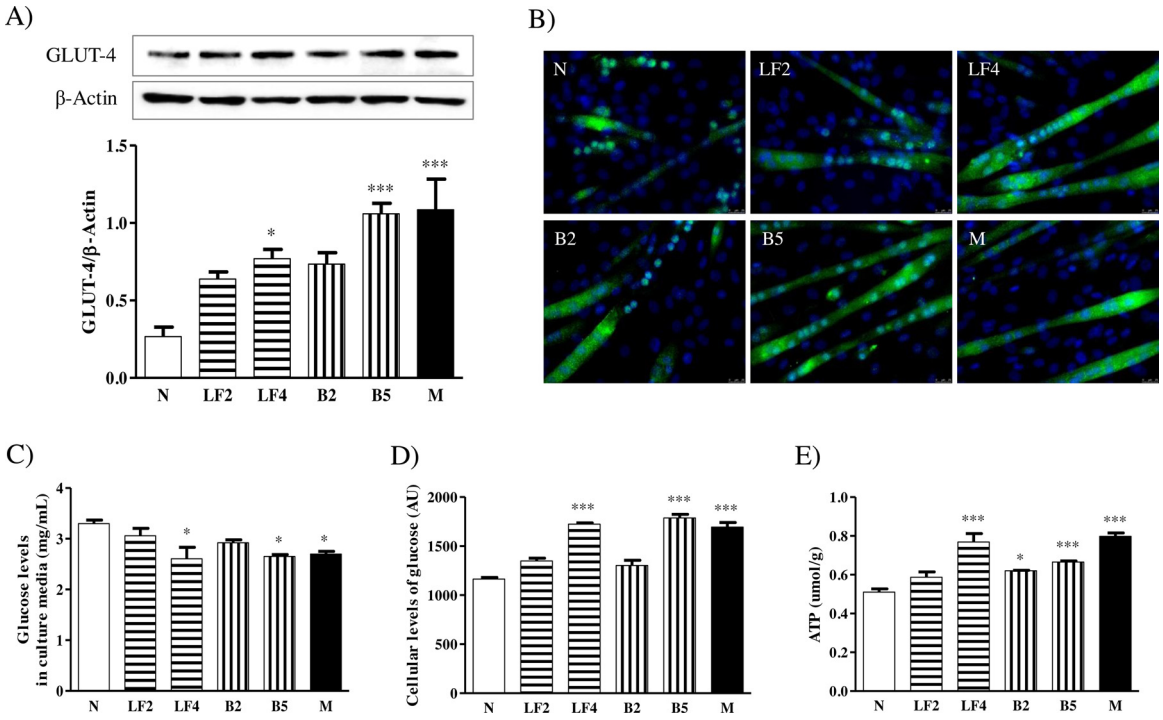


**Fig. 5.** Effect of LF extract and betaine on the expression of GLUT-4 and the glucose levels in C2C12 myotubes.

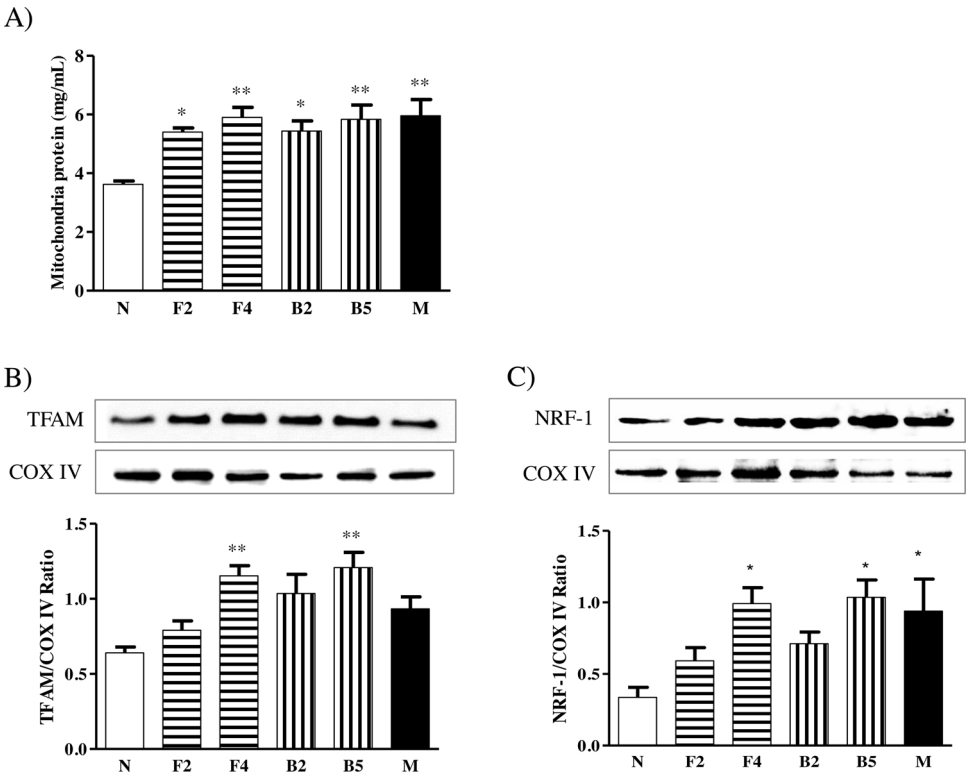


**Fig. 6.** Effect of LF extract and betaine on mitochondrial protein contents and the mitochondrial expression of TFAM and NRF-1 in C2C12 myotubes.

significantly increased after being treated with LF extract ($p < 0.05$, Fig.5A) and betaine ($p < 0.001$) at high concentrations, and this increasing feature was also found in immunocytochemical observation (Fig.5B). In addition, LF extract and betaine significantly decreased the glucose levels in the culture medium ($p < 0.05$, Fig.5C) and increased the cellular levels of glucose ($p < 0.001$, Fig.5D), compared with non-treated cells. LF extract and betaine significantly increased the ATP contents ($p < 0.001$, Fig.5E) in the myotubes. Metformin also significantly increased the GLUT-4 protein expression ($p < 0.001$) and translocation on plasma membrane, with an increase of the cellular glucose levels ($p < 0.001$) and ATP contents ($p < 0.001$), compared to non-treated cells. These results indicate that LF extract and betaine stimulate glucose uptake in myotubes through the up-regulation of the GLUT-4 translocation and improve ATP production.

(A) The expression of GLUT-4 protein was determined by Western blot. Metformin (2.5 mM) was used as a positive control, 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. (B) The expression of GLUT-4 protein was observed by fluorescence microscopy after staining with DAPI and anti-GLUT4 antibody (original magnification = 400×). Green, GLUT4-positive cells; blue, DAPI-positive nuclei. The levels of glucose in a culture medium (C) and in the cells (D) 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 (E). All data were presented as the means ± SEM of three independent experiments. N, normal cell; LF, LF extract; B, betaine; and M, metformin.*p < 0.05and **p < 0.01vs.non-treatedcells.

### 3.5. Effects of LF extract and betaine on mitochondrial biogenesis

To investigate the effects of LF extract and betaine on the mitochondrial function in muscle cells, we measured the mitochondrial protein levels and the mitochondrial expression of TFAM and NRF-1 in C2C12 myotubes. As a result, LF extract ($p < 0.05$ for 2 mg/ml and $p < 0.01$ for 4 mg/ml) and betaine ($p < 0.05$ for 2 mM and $p < 0.01$ for 5 mM) significantly increased the levels of mitochondrial protein in the myotubes, compared with non-treated cells (Fig.6A). LF extract ($p < 0.01$ for TFAM and $p < 0.01$ for NRF-1) and betaine ($p < 0.01$ for TFAM and $p < 0.05$ for NRF-1) significantly increased the mitochondrial expression of TFAM (Fig.6B) and NRF-1 (Fig.6C) at high concentrations, compared with non-treated cells. Metformin also significantly increased the mitochondrial protein level ($p < 0.01$) and NRF-1 expression ($p < 0.05$), compared with non-treated cells. These results indicate that LF extract and betaine increase the energy production in myotubes through up-regulation of mitochondrial transcription.

(A) The contents of the mitochondrial protein were measured by a Bradford assay in C2C12 myotubes. The expression of TFAM (B) and NRF-1 (C) were determined in mitochondrial factions by Western blot. Metformin (2.5 mM) was used as a positive control, and COX IV 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. All data were presented as the means ± SEM of three independent experiments. N, normal cell; LF, LF extract; B, betaine; and M, metformin.*$p < 0.05$ and **$p < 0.01$vs. non-treated cells.

### 3.6. HPLC analysis of LF extract

To analyze betaine in LF extract, we performed HPLC with a standard compound (Fig.7A). The content of betaine in LF extract was subsequently calculated by comparison with peak areas (Fig.7B). The equation of calibration curves for betaine was $y = 542.64x + 15.839$. The correlation coefficient of the calibration curve was higher than 0.9995 at concentrations between 0.0125 and 0.4 mg/ml, and the concentration of betaine in LF extract was 3.18%. The relative standard deviations of precision and repeatability were 1.34% and 2.4%, respectively.

## 4. Discussion

Lycii Fructus (LF) is called Goji, goji berry, and wolfberry, and it is the dried ripe fruit of either L. *chinense*and *L. barbarum*, which are two closely related species of boxthornin the nightshade family (Solanaceae) [13]. LF is one of the most popular traditional medicine and ingredients in tonic foods in East Asia. The record for its use as a medicinal plant is found in the Compendium of Materia Medica (BencaoGangmu, 1578 CE) in the Song's Ages [18]. Nowadays, LF and its products have become increasingly popular in the health food and food supplement industries.

LF has been reported to have various activities, including anti-oxidation [19], anti-aging [20], immunoregulation [21], anti-cancer [22], neuroprotection [23], anti-diabetes [24], anti-fatigue [25] and the improvement of hepatic functions [26]. Betaine is a major bioactive compound of LF and serves as the primary compound responsible for the quality control of crude LF extract [27]. Betaine is also studied in relation to its anti-aging activity, with strong anti-oxidant effects [25,28,29].It also improves skeletal muscle strength and power performance by increasing creatinine levels [14,30]. In addition, it is reported that betaine supplementation can help to improve body composition by inhibiting the synthesis of free fatty acids into triacylglycerides [31].However, in this study, we evaluated the beneficial effects of LF extract and betaine on muscle function through its increase of muscle differentiation and mitochondrial biogenesis in C2C12 myotubes.

Skeletal muscle is the largest tissue of the human body (40–50% of body weight) and controls physiological and pathological conditions [32]. This means that the aging process is responsible for many changes in body composition, particularly a loss of skeletal muscle mass and dysfunction, which are associated with diverse metabolic disorders, such as sarcopenia, obesity and diabetes mellitus [33]. Muscle tissues contain satellite cells as skeletal muscle-derived stem cells, which lie quiescent underneath the basal membrane and make up the necessary stem cell pool for myogenesis [34]. When satellite cells are activated, e.g., after an injury, they enter the myogenic differentiation process. During differentiation, satellite cells and myoblasts transverse a strict sequential expression pattern of different myogenic regulatory factors (MRFs), such as MyoD, Myf5, myogenin, and MRF4 and structural muscle proteins, including MyHC [35]. MyHC is the motor protein of muscle thick filaments and is a specific marker of maturation [36]. In our study, treatments of LF extract (4 mg/ml) and betaine (2 and 5 mM) in C2C12 myotubes significantly increased the expression of MyHC in both mRNA and protein levels, with morphological changes. This result indicates that LF extract and betaine can stimulate myoblasts differentiation in the myotubes in muscle.

Mitochondrial biogenesis is enhanced in response to a metabolic shift, such as energy requirements increasing the oxidative stimulus [37]. The myotube is a highly metabolically active cell type, which is responsible for myogenes is from myoblast or pre-existing myotube differentiation. This differentiation process requires metabolic demands, leading to an activation of mitochondrial biogenesis through the coordinated expression of nuclear and mitochondrial genes, which are regulated by a network of transcription factors, such as PGC-1α, NRF-1, TFAM, and Sirt-1. These events result in an increase in mitochondria mass and energy production, particularly skeletal muscle. Some diseases do not have exclusively a mitochondrial origin, but they might have an important mitochondrial component, both in their onset and development. Additionally, mitochondrial biogenesis is primarily regulated by the key transcription factor, PGC-1α, which is itself under the control of AMPK and Sirt-1 [38]. Moreover, mitochondrial biogenesis provides cellular chemical energy in the form of ATP, which stimulates AMPK activation and further mediates the increase of the GLUT-4 expression and regulation of fatty acid oxidation via ACC phosphorylation [39].In our study, the treatment of LF extract and betaine significantly promoted mitochondrial biogenesis in myotubes through the up-regulation of the cellular and mitochondrial expression of PGC-1α, TFAM, NRF-1, and Sirt-1 with the activation of the AMPK/ACC signaling pathway.

C2C12 cells, the mouse skeletal muscle cell line, have been widely used as a model to investigate the effects of various agents not only in myoblasts, a non-differentiated state on myogenic differentiation, but also in myotubes, a differentiated state on insulin dependent or independent mechanisms of glucose uptake and GLUT-4 expression [40,41]. In our study, C2C12 myotubes were employed to investigate the effects of LF extract and betaine on glucose uptake and mitochondrial biogenesis in differentiated state and found that LF extract and

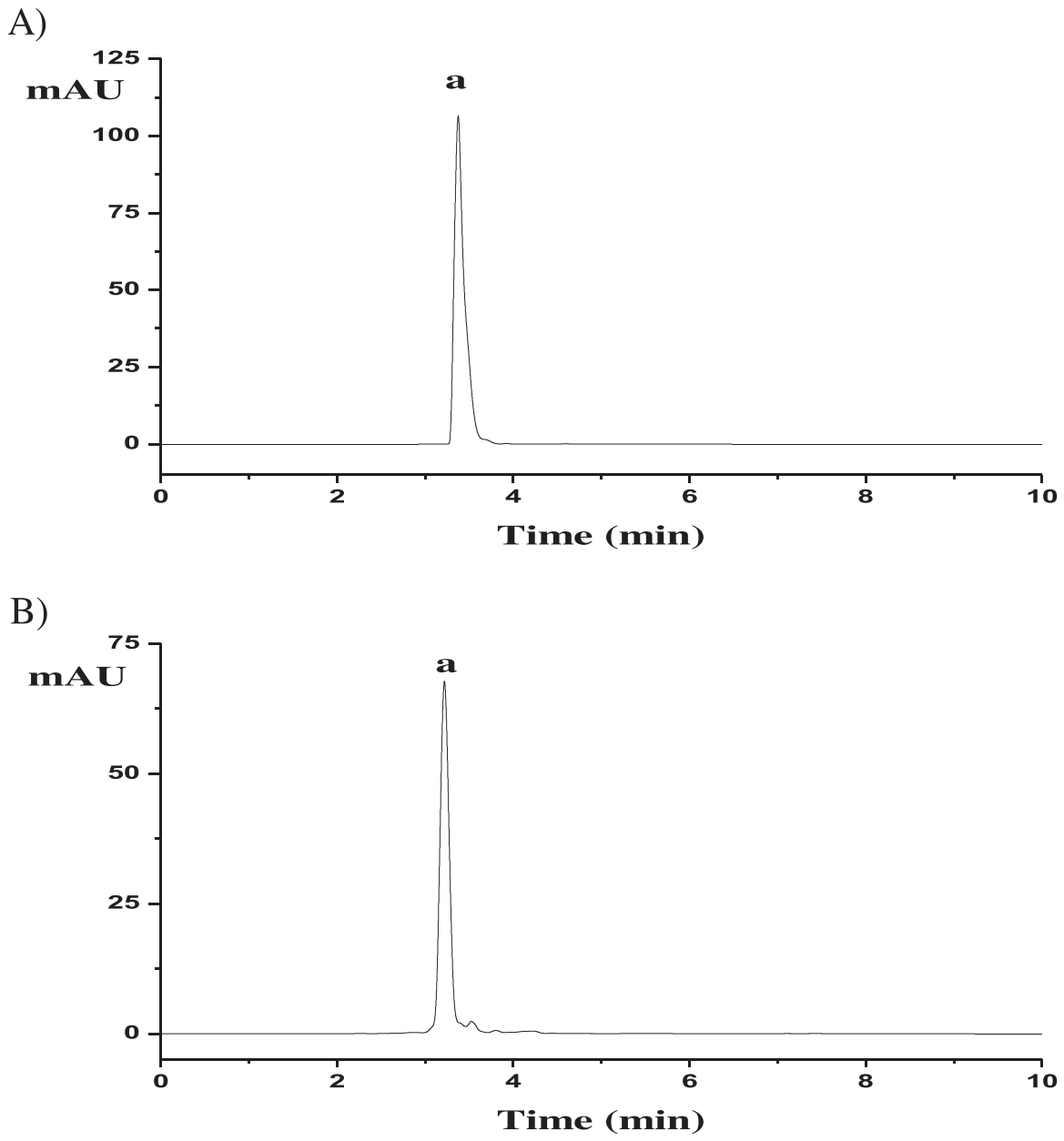


**Fig. 7.** HPLC analysis of betaine in LF extract.
(**A**) Betaine as a standard compound, and (**B**) betaine in the LF extract water extract. a, betaine (retention time: 3.375 min).

betaine promote glucose uptake with GLUT-4 expression, and also directly increased ATP production. These results indicate that LF extract and betaine can help to stimulate glucose utilization in muscle cells and to improve energy production by increasing the mitochondrial biogenesis. However, additional studies needed to reveal whether LF extract and betaine have effects on the regulation of MRFs and insulin dependent mechanism of glucose uptake during myoblasts differentiation into myotubes.

Betaine is a major biological active component in LF extract and was analyzed by HPLC. In the future, we will investigate the effects of LF extract and betaine on muscle dysfunction to determine whether they have therapeutic potential for the treatment of muscle disorders, such as sarcopenia, obesity and diabetes.

## 5. Conclusions

Our data showed that LF extract and betaine stimulate myoblasts differentiation in myotubes with the increase of the MyHC expression in C2C12 cells. LF extract and betaine improve glucose uptake and ATP production in the myotubes through the up-regulation of the mitochondrial biogenesis regulating factors, PGC-1α, NRF-1, TFAM, and Sirt-1, and the activation of the AMPK/ACC signaling pathway. Thus, this finding suggests that LF extract and betaine, by improving energy metabolism, have beneficial effects for the promotion of skeletal muscle function.

## Author contributions

Y.-K.P. and H.W.J. designed the study. J.M., H.W.J., X.M., S.Y.K., J.Z. performed the experiment and conducted the statistical analysis. J.M. and H.W.J. wrote the manuscript. All authors revised the manuscript and approved the final version.

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## Declaration of Competing Interest

The authors declare no conflict of interest.

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