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Single Clove Garlic Extract Attenuates Dexamethasone-Induced C2C12 Myotube Atrophy through Akt-Related Signalling

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Abstract

The number of older adults with skeletal muscle loss or sarcopenia is gradually increasing. In response, the interest in exploring novel drugs and supplements to attenuate muscle atrophy is growing. This research aimed to investigate the effect of single clove garlic extract (SGE) on muscle cell proliferation, differentiation, and atrophy. SGE was prepared using the maceration method. C2C12 skeletal myoblasts were treated with SGE before being subjected to MTT assay and flow cytometry to determine cell viability and proliferation. Myoblast differentiation was induced to form multinucleated myotubes, which were then treated with 10 µM dexamethasone (DEX) for 24 h to induce atrophy, in the presence or absence of SGE. After treatment, genes and proteins related to myotube atrophy were quantified using real-time PCR, immunostaining, and Western blot. Treatment with SGE (5 - 40 µg/mL) had no effect on cell viability, proliferation, or differentiation of C2C12 myoblasts. However, in myotubes treated with DEX, diameter and the expression of myosin heavy chain (MHC), MyoD, and myogenin proteins were all significantly reduced; pretreatment with SGE significantly attenuated these effects. SGE pretreatment also significantly increased the phosphorylation of Akt, P70S6K, and β-catenin, and significantly suppressed the expression of atrogin-1 compared to DEX treatment alone. Furthermore, the genes *PGC-1α*, *NRF-1*, and *TFAM* were significantly down-regulated in myotubes treated with DEX compared to control. Pretreatment with SGE significantly alleviated this effect. Thus, this study shows that SGE prevents muscle protein degradation and muscle atrophy induced by DEX via Akt protein phosphorylation and subsequent suppression of the atrogin-1 protein.

Keywords: C2C12 myoblasts, Muscle atrophy, Myogenesis, Myotube, Sarcopenia, Garlic extract, Skeletal muscle

Introduction

Skeletal muscle is a specialised tissue responsible for body movement and metabolism. Healthy skeletal muscle maintains mass by balancing protein synthesis and protein degradation. Various conditions, including ageing, lead to a gradual loss of this muscle mass [1,2]. Muscle atrophy in older people, or sarcopenia, is

characterised by reduced muscle mass, and strength, and physical functions which contribute to disability in this population. This problem places a heavy economic burden on society. Two biomarkers of muscle wasting have been identified in sarcopenia: increases in the proteins muscle atrophy F-box (atrogin-1) and muscle RING finger-1 (MuRF-1) [3]. Both atrogin-1 and MuRF-1 promote ubiquitin-mediated protein degradation, leading to skeletal muscle protein loss. Therefore, an increase in either atrogin-1 or MuRF-1 protein exacerbates skeletal muscle atrophy [4]. With growing ageing populations, interest is rising in the exploration of new interventions and supplements to prevent muscle atrophy and increase muscle protein synthesis.

Garlic (*Allium sativum*) is a widely used herb in food preparation, and is also common in traditional medicine in Asian and other countries for the treatment of various diseases [5]. The major active compounds in garlic are reported to be organosulfurs, such as diallyl thiosulfonate (allicin), diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS), E/Z-ajoene, S-allyl-cysteine (SAC), and S-allyl-cysteine sulfoxide (alliin) [6]. For nutraceutical usages, single clove garlic extract (SGE) is preferable due to its 5 - 6-fold higher content of bioactive compounds than multi-clove garlic [7]. Single clove garlic contains diverse bioactive compounds, including organic sulfides, saponins, phenolic compounds, and polysaccharides [6]. Numerous studies have demonstrated multimodal biological functions of garlic extract, including anti-oxidant, anti-cancer, anti-inflammatory, anti-diabetes, anti-obesity, anti-thrombotic, and anti-atherosclerotic effects, alongside decreased blood lipids, blood glucose modulation, cardiovascular disease treatment, and stimulation of the immune system [6,8].

Age-related cellular oxidative stress has been linked to the atrophy of skeletal muscle [1,2]. Given the anti-oxidant and other varied biological activities of SGE [6,8], research has suggested its potential for preventing muscle atrophy. Previous research reports that oral administration of garlic supplement upregulated muscle mitochondrial biosynthesis in skeletal muscle after exercise [9] and prevented skeletal muscle damage by reducing inflammation and apoptosis [10]. In addition, it has been reported to alleviate skeletal muscle loss and enhance myogenic differentiation in obese subjects via the modulation of Akt/mTOR/p70S6K signalling [11]. Moreover, a specific compound present in garlic extract has also been reported to exert a beneficial effect for the treatment and prevention of muscle atrophy. Saponins are reported to protect against H_2O_2 and inhibit myoblast growth and DNA damage by scavenging intracellular reactive oxygen species (ROS) [12]. DATS further demonstrated a protective effect against oxidative stress-induced DNA damage and apoptosis in skeletal muscle myoblast cells through ROS scavenging [13]. Additionally, Z-ajoene has been shown to ameliorate muscle atrophy by regulating myokine secretion in the JAK/STAT3 and SMADs/forkhead box O (FoxO) signalling pathways, aiding the preservation of muscle mass. It also functions to degrade myoprotein under muscle wasting and stimulate myogenesis [14]. Moreover, garlic extract has been reported to inhibit the expression of the protein FoxO [14], which acts as the master regulator of muscle atrophy in ageing through the activation of the ubiquitin proteasome system, which ultimately leads to muscle protein degradation [15]. Inhibition of the activity of FoxO may reduce the expression of atrogin-1 and MuRF-1, thus preventing muscle atrophy [16].

We hypothesise that the numerous biologically active compounds found in SGE—each with beneficial individual effects on muscle cells—may exert a synergistic therapeutic effect for the prevention and treatment of muscle atrophy and sarcopenia in older adults. Therefore, the present study aimed to explore the synergistic effect and possible mechanisms of SGE on muscle cell proliferation, differentiation, and myotube atrophy prevention.

Materials and methods

Single clove garlic extract preparation

Single clove garlic (*Allium sativum* L.) was collected from Temanggung, Central Java, Indonesia. SGE was prepared using the maceration method with an ethanol solvent, following previous reports with little modification [17-19]. This extraction protocol has been reported to extract the organosulfur compounds validated by Fourier transform infrared (FTIR) spectroscopy and high-performance liquid chromatography (HPLC). Briefly, 1 kg of mashed single clove garlic was soaked in 70% ethanol at a ratio

of 1:3. It was placed in a water bath at 37 °C for 24 h with constant shaking at 100 rpm. The solution was then passed through filter paper. This step was repeated 3 times with new 70% ethanol. The 70% ethanol containing active compounds was evaporated using a rotary evaporator. The extraction yield was 27.5% (w/w). The SGE was kept at 4 °C until use, at which point it was reconstituted in dimethyl sulfoxide (DMSO) at 40 mg/mL as a stock solution. To create the working solution, the stock solution was diluted in cell culture medium and then filtered through a sterile 0.2 µm syringe filter. The final concentration of DMSO in the culture medium was 0.1%. An identical solution of 0.1% DMSO in cell culture medium without the active SGE compounds was used as a control solution.

C2C12 myoblast cell culture

A C2C12 myoblast cell line was purchased from the American Type Culture Collection (ATCC). It was maintained in growth medium (GM) composed of Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solutions (penicillin 100 U/mL, streptomycin 100 µg/mL) at 37 °C in a humidified 5% CO_2 incubator. Cell passages 10 - 15 were used. To study the cytotoxic and proliferative effects of SGE, C2C12 myoblasts were seeded at 1×10^4 cells/well in a 96-well plate and cultured in low serum medium or GM containing SGE at 0 - 40 µg/mL for 24, 48, and 72 h [20,21]. Cell morphology was observed and photographed. For myotube formation, C2C12 myoblasts were seeded at 3×10^5 cells/well in a 6-well plate. To induce myotube differentiation, confluent cells were switched to a differentiation medium (DM) composed of DMEM supplemented with 2% horse serum (HS) for 3 days [20,21]. The myotubes were pretreated with SGE at 0 - 40 µg/mL for 2 h before 10 µM dexamethasone (DEX) was added directly to the medium. Treatment with 10 µM DEX for 24 h has been reported to induce myotube atrophy [22]. The myotubes were further cultured under these conditions for 24 h. Myotube morphology was observed and photographed. To test for mycoplasma contamination, routine Hoechst staining was performed every month.

MTT assay

The treated cells were incubated with GM containing 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 3 h. Thereafter, the solution was discarded and replaced with 100 µL DMSO. Absorbance was measured at 570 nm using a microplate reader [20,21]. Cell viability and proliferation were normalised to a control group and reported as a percentage of the control value.

Nuclear staining

The treated cells were fixed with ice-cold methanol at −20 °C for 10 min. After several washes and rehydration with phosphate buffered saline (PBS), the cells were incubated with DAPI (4′,6-diamidino-2-phenylindole) diluted in PBS for 30 min with constant agitation. Thereafter, the solution was discarded, and the cells were washed again with PBS. Nuclear density images were captured using a fluorescence microscope [20,21].

Flow cytometry

The treated cells were trypsinised and fixed in 70% ice-cold ethanol for 10 min. After several washes with PBS, the cell pellet was incubated with ribonuclease A (50 µg/mL) diluted in PBS for 30 min at 37 °C. The cells were then immediately placed on ice for 10 min. Propidium iodide (0.5 mg/mL) was directly added, and the reaction was kept in the dark for 15 min [20,21]. The cell cycle stage was determined using a BD FACSCanto flow cytometer (BD Biosciences, USA).

Immunofluorescence staining and myotube diameter measurement

The treated cells were fixed with ice-cold methanol at −20 °C for 10 min. The fixed cells were washed and rehydrated with PBS. After permeabilisation and blocking with 5% normal goat serum at room temperature (RT) for 1 h, a mouse monoclonal anti-MHC antibody was applied and incubated overnight at 4 °C. After several washes, the cells were incubated with goat anti-mouse IgG conjugated to Alexa Fluor 488 fluorescent dye for 1 h at RT. Images were captured using a fluorescence microscope [18,19]. Myotube diameter was measured using ImageJ. Myotube diameters were averaged over 6 fields per treatment, with at least ten myotubes per field

[22]. In order to blind the measurement, 3 observers were assigned to perform measurements.

RNA isolation and real-time polymerase chain reaction (PCR)

The treated cells were subjected to total RNA isolation using Trizol. The RNA was treated with DNase to remove contamination before conversion to cDNA using a cDNA synthesis kit. Real-time PCR was performed using the Luna qPCR master mix, and relative gene expression was calculated using the comparative Ct method ($2^{-\Delta\Delta Ct}$) normalised to GAPDH [20,21]. The oligonucleotide primers used in this experiment were nuclear respiratory factor 1 (NRF-1) Fw: GGCAACAGTAGCCACATTGGCT, Rw: GTCTGGATGGTCATTTCACCGC; peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) Fw: GAATCAAGCCACTACAGACACCG, Rw: CATCCCTCTTGAGCCTTTCGTG; transcription factor A mitochondrial (TFAM) Fw: GAGGCAAAGGATGATTCGGCTC, Rw: CGAATCCTATCATCTTTAGCAAGC; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Fw: TCAATGAAGGGGTCGTTGAT, Rw: CGTCCCGTAGACAAAATGGT [23].

Protein extraction and Western blot analysis

The treated cells were subjected to protein extraction using a radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitors. Equal amounts of proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and were then transferred to a polyvinylidene fluoride membrane. The membrane was blocked for non-specific binding with 5% goat serum for 1 h before incubation with the desired primary antibody on a rocking table overnight at 4 °C. After washing, the membrane was incubated with an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody at RT for 1 h. The protein signal was visualised using the HRP-substrate by Alliance Q9 advanced chemiluminescence imaging. Band intensity was measured using ImageJ. To measure the phosphorylation of proteins, the band intensity of phosphorylated protein was normalised to total protein and then to tubulin, which served as a loading control. The intensity of each treatment group was then normalised to the control group and reported as fold-change [20,21]. Throughout the experiment, all blots were exposed in automatic mode without any saturation, which confirmed the exposure as linear.

Statistical analysis

For each experiment, at least 3 independent experiments were performed with 3 technical replicates. Data were presented as mean ± s.e.m. Statistical analysis was conducted by one-way analysis of variance (ANOVA) followed by post-hoc analysis with Tukey's test using the GraphPad Prism. The data exhibited homogeneity of variance. p-values < 0.05 were considered statistically significant.

Results and discussion

Non-toxic effects of SGE on C2C12 myoblast cells

To determine a possible toxic effect of SGE, C2C12 myoblasts were treated with SGE at different concentrations in low serum media for 24, 48, and 72 h. MTT assay revealed that treatment for these durations with SGE at 5 - 40 µg/mL had no effect on C2C12 myoblast cell viability compared to the non-treated control (**Figure 1(A)**). In addition, myoblast cells cultured in low serum media containing 5 - 40 µg/mL SGE for up to 72 h showed no detrimental cell morphology effects. Myoblast cells remained normal, showing flat, star-shaped, or fusiform morphology (**Figure 1(B)**). These findings show that SGE has no cytotoxic effect on C2C12 myoblast viability. This result is consistent with previous reports that garlic saponins are non-toxic to C2C12 myoblasts [12]. Similarly, SGE is also reported to show no effect on the viability of cardiomyocytes [24], neuronal cells [25], epithelial cells [26], or osteoblasts [27]. In addition, the unchanged myoblast morphology is another indicator of the non-toxicity of SGE. Myoblasts exposed to toxic substances have been reported to express long cytoplasmic extensions, showing a round shape, and detaching from the culture dish [20,21]. Notably, the most biologically active compound in garlic is allicin (S-(2-propenyl)-2-propene-1-sulfinothioate) [28], which has been reported to show positive effects on cell viability [29]. It has also been shown to have potent anti-cancer and anti-oxidant properties, via the activation of the Nrf2 signalling pathway [30]. Collectively, these

findings suggest that SGE is safe and appropriate for development as a drug or supplement.

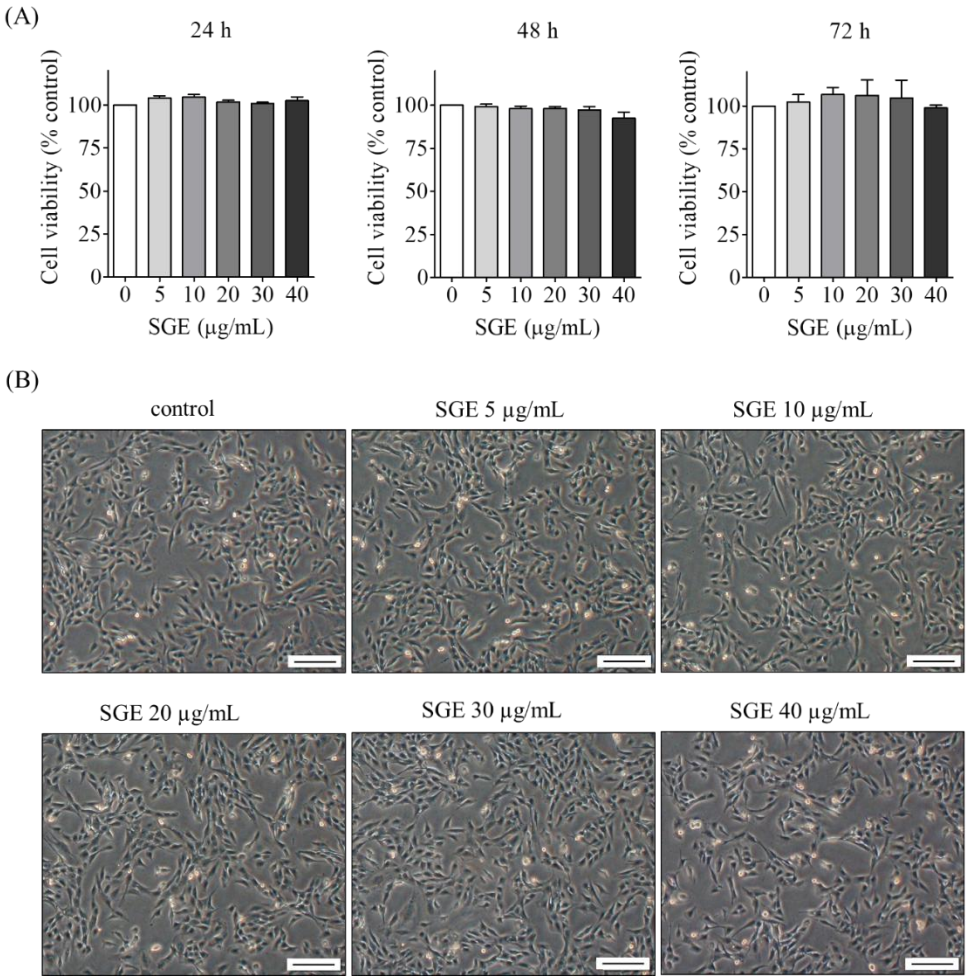

Figure 1 Non-cytotoxic effects of single clove garlic extract (SGE) on C2C12 myoblasts. Subconfluent C2C12 myoblasts were cultured in low serum media containing SGE at the indicated concentrations for 24, 48, and 72 h. The treated cells were subjected to MTT assay (A) and morphology photography at 72 h (B). Scale bar = 200 μm.

SGE did not affect C2C12 myoblast cell proliferation

To evaluate the effects of SGE on cell proliferation, C2C12 myoblasts were treated with SGE at different concentrations (5 - 40 μg/mL) in growth media for 24, 48 and 72 h. MTT assay revealed no significant increase in cell proliferation of C2C12 myoblasts treated with SGE at any concentration compared to non-treated controls at 24, 48 and 72 h (**Figure 2(A)**). In addition, no differences were observed in the total number of nuclei per area after 72 h treatment (**Figure 2(B)**), indicating equal myoblast growth rates. We further examined the effect of SGE on cell cycle distribution by flow cytometry. The percentage of cell distribution in G0/G1, S, and G2/M phases of SGE-treated groups at all doses was not significantly different compared to non-treated controls (**Figures 2(C)** and **2(D)**). Although SGE contains numerous beneficial compounds, none have been reported to stimulate proliferation of myoblasts or other cells. Allicin has been reported to have potent anti-proliferative properties in zebrafish [30]. Meanwhile, mouse fibroblasts treated with allicin from garlic extract exhibited disrupted microtubule properties, leading to the inhibition of cell proliferation [31]. In addition, diallyl polysulfides, which are derivatives of allicin, have been shown to inhibit cell proliferation by stimulated cell-cycle arrest in HCT116 human colon cancer cells [32]. Several forms of garlic, from both water and alcohol extracts, have also been shown to

have antiproliferative activity in H520, H1975, and A549 lung cancer cell lines [33]. However, our data indicated no adverse effect of SGE on C2C12 myoblast proliferation. This discrepancy is possibly due to different cell types having different sensitivities and physiological responses to garlic extract.

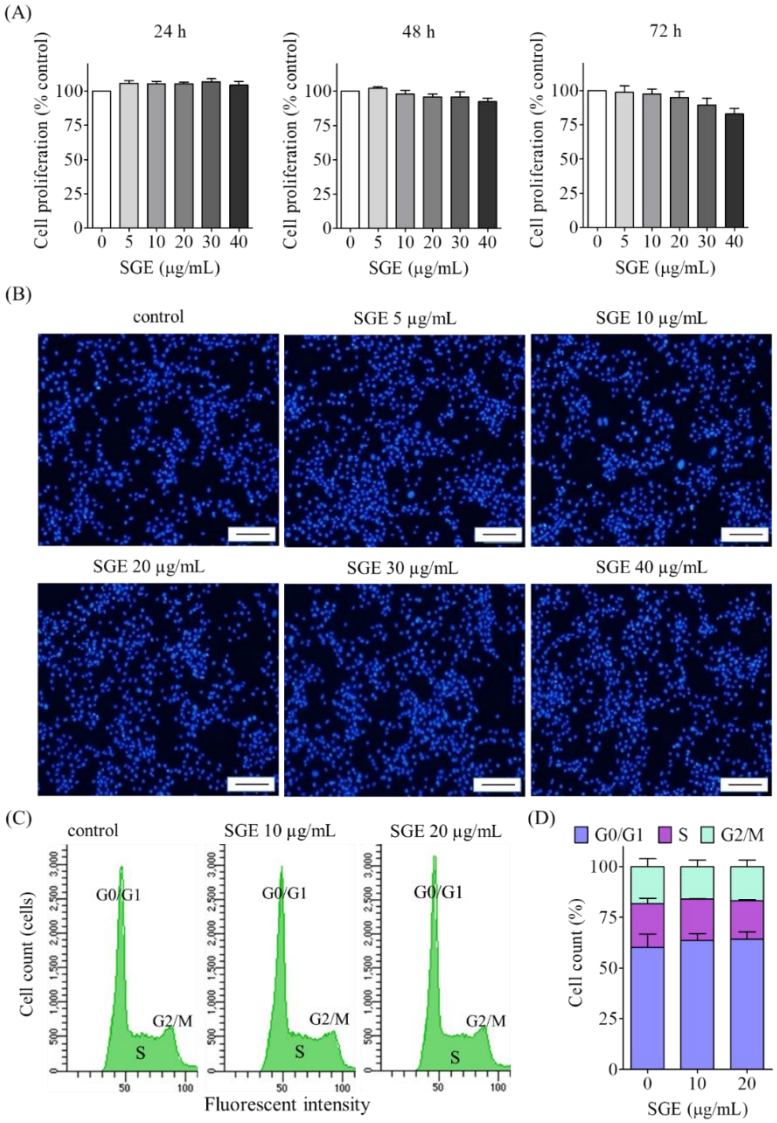

Figure 2 Effects of single clove garlic extract (SGE) on C2C12 myoblast cell proliferation. Subconfluent C2C12 myoblasts were cultured in growth media containing SGE at 5 - 40 μg/mL for 24, 48, and 72 h. The treated cells were subjected to MTT assay (A), cell density observation (B), and cell cycle determination (C). The percentage of cell cycle distributions was calculated (D). Scale bar = 200 μm.

Effect of SGE on C2C12 myoblast cell differentiation

To assess whether SGE enhanced myoblast cell differentiation, confluent C2C12 myoblasts were cultured in DM containing SGE at 5 - 40 μg/mL for 72 h. Phase contrast microscopy and MHC immunostaining revealed numerous elongated and multinucleated myotubes at all doses of SGE treatment. However, there were no significant differences in either the number or size of myotubes for any dose of SGE compared to the non-treated control (**Figure 3(A)**). In addition, levels of MHC, MyoD, and myogenin proteins were not significantly different between SGE-treated and non-treated control groups (**Figures 3(B) - 3(E)**). Similar to myoblast proliferation, SGE did not enhance myoblast differentiation. This is in contrast to previous reports, in which garlic extract has been shown to enhance PC12 nerve cell morphological differentiation with neurite

outgrowth [34] and induce morphological differentiation of MCF7 breast cancer cells via up-regulation of eIF2-α phosphorylation [35]. Meanwhile, garlic extract decreased 3T3-L1 adipocyte differentiation through activation of AMP-activated protein kinase (AMPK) [36]. These varying results may indicate cell type-specific effects. However, garlic extract has been reported to possess estrogenic activity [37], and phytoestrogen has been reported to enhance myoblast differentiation [38-40]. Thus, the correlation between estrogenic activity in garlic extract and myoblast differentiation requires further investigation.

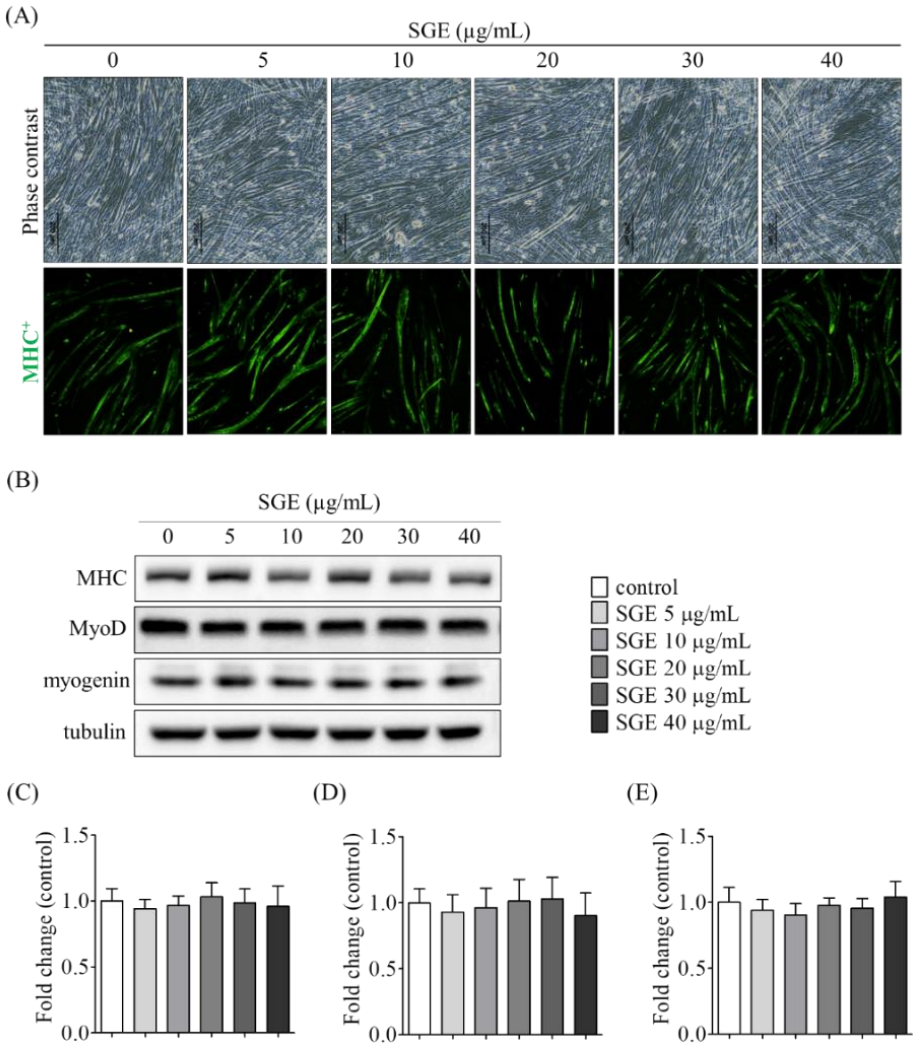

Figure 3 Effects of single clove garlic extract (SGE) on C2C12 myoblast differentiation. Confluent C2C12 myoblasts were cultured in differentiation media containing SGE at 0 - 40 μg/mL for 72 h. The differentiated myotubes were stained with anti-myosin heavy chain (MHC, green) and photographed (A) or subjected to Western blot with anti-MHC, anti-MyoD, and anti-myogenin antibodies (B). The band intensities of MHC (C), MyoD (D), and myogenin (E) were measured. Scale bar = 200 μm.

SGE protects myotubes against DEX-induced atrophy

DEX is commonly used to induce muscle atrophy in vitro through a similar mechanism to that observed in sarcopenia. We therefore aimed to determine the protective effect of SGE against DEX-induced myotube atrophy. The endpoint markers of myotube atrophy in this experiment were decreased MHC protein expression, decreased myotube size, and increased atrogin-1 protein expression. Multinucleated myotubes were treated with DEX (10 μM) in the presence or absence of SGE for 24 h. Visual inspection of immunofluorescence staining revealed that DEX treatment produced smaller myotubes than control conditions, but this effect was reversed by pretreatment

with SGE (**Figure 4(A)**). Measurement confirmed that DEX-treated myotubes were significantly smaller than controls, and that SGE pretreatment at 20 - 40 μg/mL prevented significant DEX-induced myotube atrophy (**Figure 4(B)**). In addition, the levels of MHC, MyoD, and myogenin—important proteins for myotube growth and development—were altered, consistent with the myotube size, being significantly decreased after DEX treatment compared to the control group. Pretreatment with SGE at 10 - 40 μg/mL significantly alleviated the decreased expression of these proteins (**Figures 4(C) - 4(F)**). It is noteworthy that our findings are the first report about the preventive effect of SGE on DEX-induced myotube atrophy. Prolonged administration of DEX, a corticosteroid, produces muscle atrophy by increasing the activity of 2 major muscle-specific ubiquitin ligases, atrogin-1 and MuRF-1, via the upregulation of the FoxO transcription factor [41,42]. It also induces cellular oxidative stress and mitochondrial dysfunction, further contributing to muscle atrophy [24,43]. Our findings are congruent with the previous reports on natural extracts of *Valeriana fauriei,* which possess anti-oxidant activity and can prevent myotube atrophy [44]. Thus, prevention of DEX-induced myotube atrophy may arise from the anti-oxidative activity of SGE [33,45].

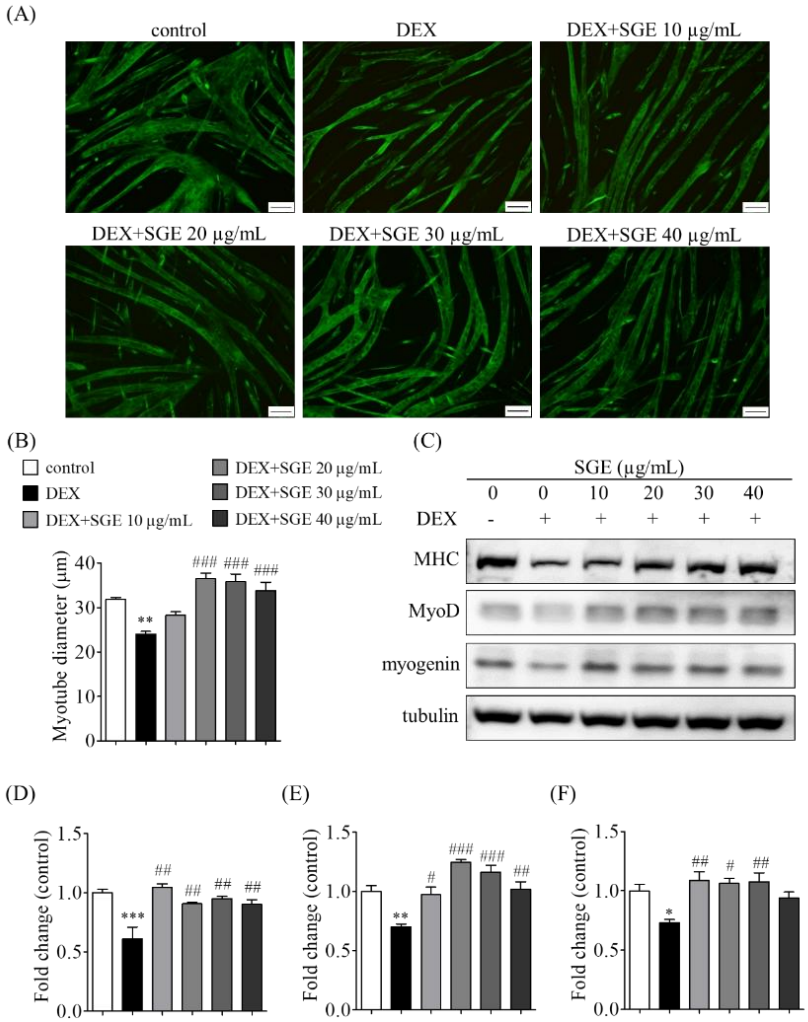

Figure 4 Single clove garlic extract (SGE) protects myotubes against dexamethasone (DEX)-induced atrophy. Confluent C2C12 myoblasts were cultured in differentiation media for 72 h. The differentiated myotubes were pretreated with SGE (10 - 40 μg/mL) for 2 h before being exposed to DEX for 24 h. The treated myotubes were stained with anti-MHC antibody (green) (A), and myotube size was measured (B). The treated myotubes were subjected to Western blot with anti-MHC, anti-MyoD, and anti-myogenin antibodies (C). The band intensities of MHC (D), MyoD (E), and myogenin (F) were measured. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to control, and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared to the DEX-treated group. Scale bar = 100 μm.

SGE alleviates DEX-induced myotube atrophy associated with increased Akt phosphorylation

To determine possible mechanisms of the protective effect of SGE against DEX-induced myotube atrophy, we further investigated the expression of the Akt protein and its downstream signalling molecules; phosphorylation of Akt is reported to be involved with both suppression of muscle protein degradation and stimulation of muscle protein synthesis. Western blot revealed significantly increased expression of atrogin-1 in DEX-treated myotubes compared to the control group. In addition, DEX induced significant decreases in the expression of the signalling molecules p-Akt, β-catenin, and p-P70S6K. Meanwhile, pretreatment with 20 μg/mL SGE significantly mitigated the downregulation of these proteins by DEX (**Figures 5(A) - 5(E)**). These results were consistent with myotube morphology and size (**Figures 5(F)** and **5(G)**). In addition, our findings indicated that SGE stimulated the phosphorylation of Akt and P70S6K and suppressed atrogin-1 expression related to DEX-induced muscle atrophy. These results are similar to previous reports on various extracts—including flavonoid dihydromyricetin [46], fucoxanthine [47], ginseng [22], *V. fauriei* [44], *Salvia plebeian,* and rosemarinic acid [48]—which can activate p-Akt, p-mTOR, and p-P70S6K, and exhibit beneficial effects in attenuating DEX-induced muscle atrophy. Garlic extract has previously been linked to the expression of phosphorylated Akt [49]. Activation of Akt has been reported to inhibit FoxO [50], which stimulates the expression of MuRF-1 and atrogin-1, two proteins responsible for muscle protein degradation and subsequent muscle atrophy [51]. Treatment of skeletal muscle with DEX has been reported to induce proteasomal proteolysis via MuRF-1 and atrogin-1 expression [52]. Our results show that SGE prevented DEX-induced muscle atrophy by suppressing the expression of atrogin-1, at least in part, through Akt-related signalling. Meanwhile, p-P70S6K and p-mTOR have been reported to be downstream mediators of Akt phosphorylation in skeletal muscle cells [53]. Activation of Akt triggers the mTOR signalling cascade, which stimulates protein synthesis and leads to the prevention of muscle atrophy [54]. Phosphorylation of mTOR also inhibits protein degradation-induced skeletal muscle atrophy in mice by attenuating autophagy-related pathways [52]. In addition, increased β-catenin is also important for the maintenance of muscle structure [55]. Apart from mTOR, phosphorylation of P70S6K in skeletal muscle cells has been shown to play an essential role in the regulation of protein translation, which is necessary for muscle protein synthesis [53,56]. Activation of P70S6K in skeletal muscle has also been reported as essential for skeletal muscle mass maintenance [56]. Altogether, these results suggest that prevention of DEX-induced myotube atrophy by SGE may act through the activation of Akt/P70S6K signalling pathways.

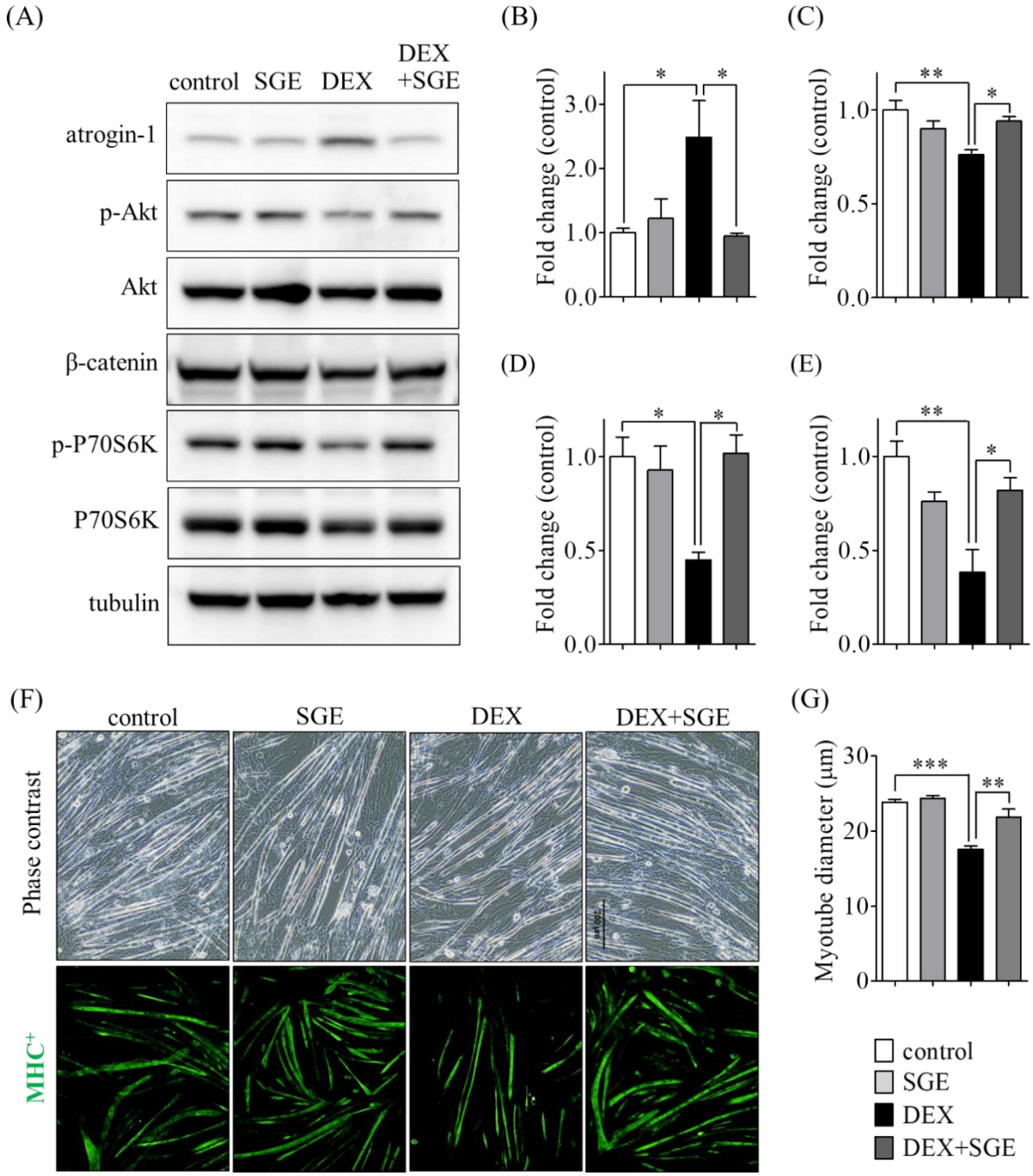

Figure 5 Single clove garlic extract (SGE) alleviates dexamethasone (DEX)-induced myotube atrophy through Akt-related signalling. Differentiated myotubes were pretreated with SGE (20 μg/mL) for 2 h before exposure to DEX for 24 h. The treated myotubes were subjected to Western blot (A). The band intensities of atrogin-1 (B), p-Akt (C), β-catenin (D), and p-P70S6K (E) proteins were measured. The treated myotubes were stained with anti-MHC antibody (green) (F), and diameters were measured (G). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to the corresponding group. Scale bar = 200 μm.

SGE modulates mitochondrial biogenesis gene expression in DEX-induced myotube atrophy

As mitochondria play an important role in muscle physiology and homeostasis, disruption of their function can contribute to skeletal muscle atrophy. We evaluated the modulation of the *PGC-1α*, *NRF-1*, and *TFAM* genes, which are implicated in mitochondrial function and muscle atrophy. These genes are also reported to be involved with the expression of phosphorylated proteins involved with muscle atrophy. Real-time PCR revealed

that differentiated myotubes treated with DEX exhibited significant downregulation of *PGC-1α*, *NRF-1*, and *TFAM* expression compared to controls. Treatment with SGE alone had no effect on the expression of these genes. However, pretreatment with SGE before 24 h-exposure to DEX significantly attenuated their DEX-induced downregulation (**Figure 6**). The modulation of myotube *PGC-1α*, *NRF-1*, and *TFAM* levels by SGE provides new insights for the prevention and treatment of muscle loss and atrophy. *PGC-1α*, *NRF-1*, and *TFAM* are involved with mitochondrial biogenesis and function [57]. SGE has been previously reported to stimulate the expression of *PGC-1α* and *NRF-1* [11], which leads to the expression of *TFAM* [58]. Expression of these genes has been linked to the ablation of FoxO-mediated atrophy in skeletal muscle [59]. In addition, *PGC-1α* has also been reported to prevent muscle fibre atrophy through the suppression of atrogin-1 and MuRF-1 expression [11,59]. In addition, the upregulation of phosphorylated Akt and P70S6K has been reported to occur through *PGC-1α*/*TFAM* signalling pathways [46]. Hence, the modulation of these genes by SGE may ultimately lead to Akt and P70S6K phosphorylation in myotubes treated with DEX, protecting them from atrophy.

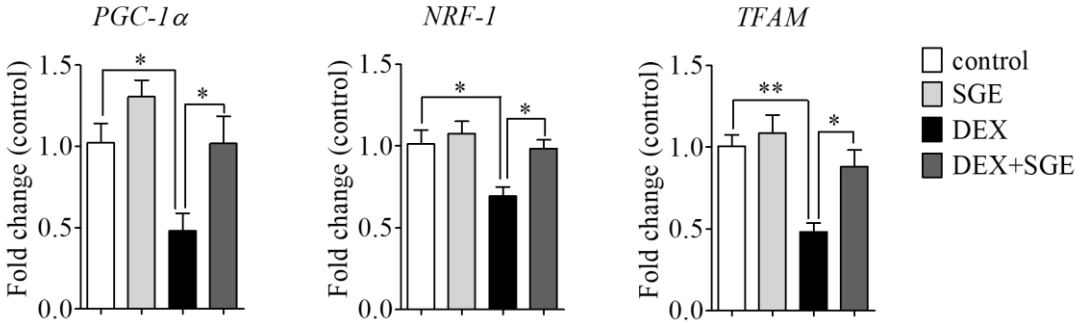

Figure 6 Single clove garlic extract (SGE) pretreatment prevents dexamethasone (DEX)-induced downregulation of *PGC-1α*, *NRF-1*, and *TFAM* genes. Confluent C2C12 myoblasts were cultured in differentiation media for 72 h. The differentiated myotubes were pretreated with SGE before exposure to DEX for 24 h. The treated myotubes underwent real-time PCR analysis. *$p < 0.05$ and **$p < 0.01$ compared to the corresponding group.

Conclusions

To the best of our knowledge, these findings provide new information, providing mechanistic insight into the preventive effect of SGE on DEX-induced myotube atrophy. These effects may act through by the upregulation of the Akt/β-catenin and Akt/P70S6K signalling pathways and of mitochondrial biogenesis genes. These results suggest that SGE has potential as a supplement for the prevention of muscle loss and atrophy in older people. However, this experiment was performed *in vitro*, which does not reflect the full physiology and response of an organism. Further studies using in vivo models and clinical trials are required. In addition, the extracted chemical constituents of SGE should be standardised for comparison across batches and studies.

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Declaration of Generative AI in Scientific Writing

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CRediT Author Statement

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