6-shogaol suppresses oxidative damage in L6 muscle cells

Jinyoung Hur, Yeonmi Lee, Chang Jun Lee, Ho-Young Park and Sang Yoon Choi*

Abstract
Ginger (Zingiber Officinale Roscoe) has been known reduce muscle pain after exercise, and 6-shogaol ((E)-1-(4-Hydroxy-3-methoxyphenyl)dec-4-en-3-one) is the major essential oil contained in ginger. In this study, the protective effect of 6-shogaol on L6 muscle cells against oxidative damage was measured. 6-shagol inhibited the damage of L6 cell induced by H2O2, and allowed the increase in mRNA and protein expression levels of intracellular HO-1 and NRF2. 6-shogaol also reduced the production of intracellular ROS. These results suggested that 6-shagol effectively inhibits oxidative damage of skeletal muscle cell.

Keywords: 6-shogaol, L6 cell, Antioxidant, Nrf2, HO-1

Introduction
Active oxygen is produced in the normal metabolic process and it performs various biological functions; but active oxygen that is excessively generated causes damage to cells and tissues [1–3]. Regular and appropriate exercise help the prevention and mitigation of hypertension, stroke, cardiovascular disease, diabetes, hyperglycemia, and cancer [4, 5]. However, strenuous exercise causes generation of excessive active oxygen and consequently oxidative damage in muscle tissue [6, 7]. The human body protects itself by generating antioxidant enzymes as a defense system against such oxidative damage but the protection ability varies depending on the persons’ age and health status.

The ginger is a zingiberaceae perennial plant of the subtropical or tropical regions, and its rhizome is edible. In recent years, it has been reported that if ingesting ginger reduces post-exercise muscle pain [8]. The typical essential oils of ginger are gingerol and shogaol (Fig. 1), and shogaol is gingerol that is converted by heat [9]. Shogaol has an antioxidant, anti-inflammatory, and the protective effect on the cranial nerve [10–14] and melanocytes [15], but studies of the effect on skeletal muscle have not been reported. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that has been considered as the main regulator of antioxidant gene such as HO-1 [16]. Oxidative stress and inflammation are main mechanisms involved in muscle atrophy [17]. In this study, we show that that three types of gingerol and 6-shogaol exhibit protective effects against damage in L6 skeletal muscle cells; cell survival rate, the amount of ATP produced, and the amount of intracellular ROS generation were measured after treating the cells with hydrogen peroxide and these compounds. In addition, the effect on mRNA and protein expression of HO-1 and NRF2, which are the intracellular antioxidant factors, was measured.

Materials and methods
Materials
6-Shogaol, 6-gingerol, 8-gingerol, and 10-gingerol were purchased from Chromadex company (Irvine, CA, USA). Dulbecco’s modification of Eagle medium (DMEM), fetal bovine serum (FBS), and Antibiotic-Antimycotic (AA) were purchased from Gibco-BRL (Carlsbad, CA, USA), and 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA) was obtained from Sigma Aldrich (St. Louis, MO, USA). Nucleospin was purchased from MACHEREY-NAGEL. (MACHEREY-NAGEL INC, PA, USA), iQ™ SYBR® Green Supermix, and iScript™ Reverse
Transcription Supermix for RT-qPCR were purchased from Bio-Rad (Hercules, CA, USA). HO-1 antibody was purchased from Enzo Life Science (Farmingdale, USA). NRF-2 antibody was purchased from Abcam (Cambridge, UK).

**Cell culture**
The L6 cell line was obtained from the Korean Cell Line Bank, and cultured by using DMEM containing 10% FBS, 1% AA at 37 °C, and 5% CO₂. Once the cells reached confluence, the medium was changed to DMEM containing 2% horse serum and 1% AA for differentiation. Experiments were undertaken 6 days after fully differentiation.

**Measurement of cell viability**
The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay method was used to measure cell viability. To investigate the protective effect of the gingerol and shogaol on muscle cells, the cultured cells were treated with the sample and 1.5 mM H₂O₂, and cultured for another 24 h. And then the absorbance at 540 nm was measured by staining with MTT (0.5 mg/ml in PBS) solution. The protective effect was expressed as a percentage by calculating the recovery ratio when treating with the sample versus the cell death ratio when treating with hydrogen peroxide.

**Real-time PCR**
Total RNA from each cell was extracted using NucleoSpin® RNA isolation kit (MACHEREY–NAGEL INC, PA, USA) and subjected to Real time-PCR using the following primers: HO-1 forward primer: AAGAGGCTA AGACCGCCTTC and reverse primer: 5′-GCAAGGCTA AGACCGCCTTC-3′; GAPDH forward primer: 5′-AGG TGTCTCCTGTGACTTC-3′ and reverse primer: 5′ctgttgctgtagacattc-3′. The cDNAs were amplified by 30 cycles of 42 °C for 45 s on a real-time PCR using a iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA). Real-time PCR was performed according to CFX Manager software using C1000 thermo cycler system (Bio-Rad, Hercules, CA, USA) which allows for real-time quantitative detection of the PCR product based on an increase in SYBR green fluorescence caused by the binding of SYBR green to double stranded DNA. For sample analysis, the threshold was set based on the exponential phase of the products, and the CT value for the samples

![Chemical structure of 6-shogaol, 6-gingerol, 8-gingerol, 10-gingerol and curcumin](image-url)
was determined. The resulting data were analyzed using the comparative CT method for relative gene expression quantification against the housekeeping gene GAPDH.

**Western blot analyses**

Cells were washed and collected with PBS. After centrifugation, the cells were lysed with Protein Extraction Solution (Elpis Biotech, Daejeon, Korea). Protein concentrations in each fraction were measured using a protein assay dye reagent and bovine serum albumin as the standard. Equal amounts of protein were electrophoresed on a 10% sodium dodecyl sulphate–polyacrylamide gel and transferred onto a nitrocellulose membrane. The membranes were blocked with 5% skim milk and incubated with primary antibodies against HO-1 (1:2000 dilution) and Nrf2 (1:1000 dilution). Subsequently, the membranes were incubated with an appropriate horseradish peroxidase-conjugated secondary antibody. Antibody detection was performed using ECL reagent (Thermo Scientific, USA) and visualized with a ChemiDoc imaging system (Bio-Rad Laboratories, Hercules, CA, USA). Band intensities were analyzed using Bio-Rad Image Lab Software.

**Measurement of active oxygen generation**

Intracellular active oxygen was dyed by using fluorescence 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA). The L6 cells were pretreated with 6-shogaol for 1 h and then treated with H₂O₂ for 30 min. The cells were treated with 3 μM of DCF-DA and then supplementary cultured for 30 min. DCF-DA positive cells were confirmed by using a fluorescence microscope (Nikon, Eclips Ti microscopy, Japan).

**Measurement of adenosine triphosphate (ATP) generation**

The amount of ATP in L6 cells was measured by using ATP bioluminescence assay kit HS II (Roche, Germany). L6 cells were seeded on 96 well plates in the concentration of 1 × 10⁵ cells/ml and cultured for 24 h. The cells were then simultaneously treated with 6-shogaol and 1.5 mM of H₂O₂. After additional culturing for 24 h, the cells were lysed and ATP bioluminescence assay was carried out. The absorbance was measured by using a luminometer (Molecular Devices, USA).

**Statistical analysis**

The data are indicated as mean ± standard deviation, and statistical significance was verified using one-way ANOVA followed by Tukey’s post hoc test. P-values less than 0.05 were considered to be statistically significant.

**Results**

**Protective effects on L6 muscle cells**

Violent exercise generates excessive active oxygen, and as a result, the muscle tissues are subjected to oxidative damage [18]. In order to determine whether gingerol and shogaol exhibit protective effect on muscle cell against oxidative damage, the protective effect on L6 cells against hydrogen peroxide-induced damage was measured. After confirming that L6 cell death was not induced by 100 μM of 6-gingerol, 8-gingerol, 10-gingerol, and 6-shogaol, L6 cells were treated for 24 h with 1, 10, and 100 μM of these materials along with 2 mM of hydrogen peroxide. 76.4% of the cells died when treated with only hydrogen peroxide, but when the cells were simultaneously treated with 6-shogaol and hydrogen peroxide, cell death inhibition
was dose dependently decreased. The cell death inhibition rate of 6-shogaol was 31.9, 47.5, and 50.7% at 1, 10, and 100 μM, respectively, and the inhibitory effect was higher than three kinds of gingerol (Fig. 2).

**Effect on HO-1 and NRF-2 expression in L6 cells**

HO-1 is an antioxidant transcription factor and NRF-2 helps the transcription of the gene related to antioxidant by entering in the nucleus because of an external stimulus [19, 20]. In order to measure the effect of 6-shogaol on the expression of HO-1 and Nrf2, the expression levels of the gene and protein were confirmed by western blot analysis and real time-PCR analysis. The protein levels of HO-1 and Nrf2 are shown in Fig. 3. As a result, the expression of HO-1 and Nrf2 protein was increased compared to the control when treated with 6-shogaol. The expression of HO-1 mRNA increased approximately 2.5 times compared to the control with treatment with 10 μM of 6-shogaol. The expression of Nrf2 mRNA was increased 1.7 times compared to the control with 6-shogaol treatment (Fig. 4). These results suggested that the antioxidative effects of 6-shogaol mediated by upregulation of HO-1 and Nrf2.

**ROS measurement**

In order to confirm that 6-shogaol inhibits the production of ROS in L6 cell, the amount of intracellular ROS was measured using the fluorescent dye DCF-DA. L6 cells were simultaneously treated with 6-shogaol and H₂O₂ for 1 h, and then were stained for 30 min by ROS-sensitive fluorescent dye 2',7'-dichlorofluorescein diacetate (DCF-DA, Sigma, USA) and observed by a fluorescence microscope. As a result, when treating with only H₂O₂, the production of intracellular ROS was significantly increased. However, when treating along with 6-shogaol, the production of intracellular ROS was decreased (Fig. 5).

**Effect on ATP production in muscle cells**

ATP is necessary for the survival of cells [21]. The effect of 6-shogaol on production of ATP in L6 cells was measured and hydrogen peroxide-induced reduction of ATP production in L6 cells was effectively inhibited when treating with 6-shogaol (Fig. 6).

**Discussion**

Gingerol and shogaol are pungent component in ginger. Gingerol is the most abundant in fresh ginger and shogaol is produced by dehydrated from gingerol [9].
6-shogaol has been reported to have higher radical scavenging effect and anti-inflammatory effect than gingerol [22]. In addition, 6-shogaol acutely relaxes airway smooth muscle and attenuates human artery smooth muscle cells calcification [23, 24]. It has been reported that treatment of ginger extracts resulted in improvement of cellular differentiation of myoblast by cell cycle regulation [25]. However, effect of 6-shogaol in differentiated skeletal muscle cells has not been studied so far. Skeletal muscle can respond rapidly to other environmental changes by modifying of genes involved muscle structure, energy metabolism. Changes of ROS and inflammation in skeletal muscle cell by exercise and energy metabolism affected muscle atrophy [26–28]. In this study, 6-shogaol significantly inhibited hydrogen peroxide induced cell death in L6 skeletal muscle cells, treatment with 6-gingerol, 8-gingerol and 10-gingerol had no inhibitory effect. Moreover, 6-shogaol inhibited the production of intracellular ROS by increasing mRNA and protein expression of HO-1 and Nrf2, which control the expression of the antioxidant enzyme. These results suggested that the 6-shogaol might be able to inhibit muscle atrophy in differentiated muscle damage as well as muscle regeneration which related muscle development. The in vivo study of 6-shogaol requires, and will be processed in further investigation.

In summary, 6-shogaol significantly inhibited hydrogen peroxide induced cell death in L6 skeletal muscle cells, treatment with 6-gingerol, 8-gingerol and 10-gingerol had no inhibitory effect. The 6-shogaol inhibited the production of intracellular ROS. Moreover, 6-shogaol increased mRNA and protein expression of HO-1 and Nrf2, which control the expression of the antioxidant enzyme. These results suggested that the 6-shogaol might be able to inhibit oxidative stress induced muscle damage.

Authors’ contributions
Performed the experiments: JH, YL, CJL. Data analysis: JH, YL, HYP, SYC. Wrote the manuscript: SYC, JH. Designed and supervised the study: SYC. All authors read and approved the final manuscript.

Funding
This study was supported by the Korea Food Research Institute (E0186701-03).

Availability of data and materials
The datasets used in this study are available from the corresponding author on reasonable request.

Competing interests
All authors declare that there is no competing interests.

Received: 29 July 2020 Accepted: 11 September 2020 Published online: 19 September 2020

References
1. Wei H, Cong X (2018) The effect of reactive oxygen species on cardiomyocyte differentiation of pluripotent stem cells. Free Radic Res 11:1–9
2. Chung S, Dzeja PP, Faustino RS, Perez-Terzic C, Behfar A, Terzic A (2007) Mitochondrial oxidative metabolism is required for the cardiac differentiation of stem cells. Nat Clin Pract Cardiovasc Med 4:560–567
3. Mandal S, Lindgren AG, Srivastava AS, Clark AT, Banerjee U (2011) Mitochondrial function controls proliferation and early differentiation potential of embryonic stem cells. Stem Cells 29:486–495
4. Leggio M, Fusco A, Limongelli G, Sgorbini L (2018) Exercise training in patients with pulmonary and systemic hypertension: a unique therapy for two different diseases. Eur J Intern Med 47:17–24
5. Rzchorzek W, Zhang H, Buckley BK, Hua K, Pomp D, Faber JE (2017) Aerobic exercise prevents rarefaction of pial collaterals and increased
stroke severity that occur with aging. J Cereb Blood Flow Metab 37:3544–3555
6. Howatson G, Van Someren KA (2008) The prevention and treatment of exercise-induced muscle damage. Sports Med 38:483–503
7. Aguiló A, Tauler P, Fuentespina E, Tur JA, Córdova A, Pons A (2005) Antioxidant response to oxidative stress induced by exhaustive exercise. Physiol Behav 84:1–7
8. Black CD, O’Connor PJ (2010) Acute effects of dietary ginger on muscle pain induced by eccentric exercise. Phytother Res 24:1620–1626
9. Ko MJ, Nam HH, Chung MS (2019) Conversion of 6-gingerol to 6-shogaol in ginger (Zingiber officinale) pulp and peel during subcritical water extraction. Food Chem 270:149–155
10. Kim S, Kwon J (2013) [6]-shogaol attenuates neuronal apoptosis in hydrogen peroxide-treated astrocytes through the up-regulation of neurotrophic factors. Phytother Res 27:1795–1799
11. Ohnishi M, Ohsita M, Tamaki H, Marutani Y, Nakayama Y, Akagi M, Miyata M, Maehara S, Hata T, Inoue A (2018) Shogaol but not gingerol has a neuroprotective effect on hemorrhagic brain injury: contribution of the α, β-unsaturated carbonyl to heme oxygenase-1 expression. Eur J Pharmacol 842:33–39
12. Xu Y, Bai L, Chen X, Li Y, Meng X, Zhang Q (2018) 6-Shogaol ameliorates diabetic nephropathy through antioxidant, hyperlipidemic,
13. Na JY, Song K, Lee JW, Kim S, Kwon J (2016) Pretreatment of 6-shogaol attenuates oxidative stress and inflammation in middle cerebral artery occlusion-induced mice. Eur J Pharmacol 788:241–247
14. Kou X, Wang X, Ji R, Liu L, Qiao Y, Lou Z, Ma C, Li S, Wang H, Ho CT (2018) Occurrence, biological activity and metabolism of 6-shogaol. Food Funct 9:1310–1327
15. Townsend EA, Siviski ME, Zhang Y, Xu C, Hoonjan B, Emala CW (2012) Effects of ginger and its constituents on airway smooth muscle relaxation and calcium regulation. Am J Respir Cell Mol Biol 48:157–163
16. Chen TC, Yen CK, Lu YC, Shi CS, Hsieh RZ, Chang SF, Chen CN (2020) The antagonism of 6-shogaol in high-glucose-activated NLRP3 inflammasome and consequent calcification of human artery smooth muscle cells. Cell Biosci 10:5
17. Anderson LJ, Liu H, Garcia JM (2017) Sex differences in muscle wasting. Adv Exp Med Biol 1043:153–197
18. Pingitore A, Lima GP, Mastorci F, Quinones A, Iervasi G, Vassalle C (2015) Exercise and oxidative stress: potential effects of antioxidant dietary strategies in sports. Nutrition 31:916–922
19. Ogborne RM, Rushworth SA, Charalambos CA, O’Connell MA (2004) Haem oxygenase-1: a target for dietary antioxidants. Biochem Soc Trans 32:1003–1005
20. Baird L, Dinkova-Kostova AT (2011) The cytoprotective role of the Keap1-Nrf2 pathway. Arch Toxicol 85:241–272
21. Fei F, Zhu DL, Tao LJ, Huang BZ, Zhang HH (2015) Protective effect of ATP on skeletal muscle satellite cells damaged by H2O2. J Huazhong Univ Sci Technol Med Sci 35:76–81
22. Dugasan S, Pichika MR, Natarajah VO, Bajiypalli MK, Tandra S, Korlakuntha JN (2010) Comparative antioxidant and anti-inflammatory effects of [6]-gingerol, [8]-gingerol, [10]-gingerol and [6]-shogaol. J Ethnopharmacol 127:515–520
23. Townsend EA, Siviski ME, Zhang Y, Xu C, Hoonjan B, Emala CW (2012) Effects of ginger and its constituents on airway smooth muscle relaxation and calcium regulation. Am J Respir Cell Mol Biol 48:157–163
24. Chen TC, Yen CK, Lu YC, Shi CS, Hsieh RZ, Chang SF, Chen CN (2020) The antagonism of 6-shogaol in high-glucose-activated NLRP3 inflammasome and consequent calcification of human artery smooth muscle cells. Cell Biosci 10:5
25. Sahardi NFNM, Jaafar F, Mariam Nordin FM, Makpo S (2020) Zingiber Officinale Roscoe prevents cellular senescence of myoblast in culture and enhances muscle regeneration. Evid Based Complement Alternat Med 1787342:1–13
26. Miller CJ, Gounder SS, Kannan S, Goutham K, Muthusamy VR, Firpo MA, Symons JD, Paine R, Hoidal JR, Rajasekaran NS (2012) Disruption of Nrf2/ARE signaling impairs antioxidant mechanisms and promotes cell degradation pathways in aged skeletal muscle. Biochim Biophys Acta 1822:1038–1050
27. Safdar A, deBeer J, Tarnopolsky MA (2010) Dysfunctional Nrf2-Keap1 redox signaling in skeletal muscle of the sedentary old. Free Radic Biol Med 49:1487–1493
28. Narasimhan M, Hong J, Atieno N, Muthusamy VR, Davidson CJ, Abu-Rmaileh N, Richardson RS, Comes AV, Hoidal JR, Rajasekaran NS (2014) Nrf2 deficiency promotes apoptosis and impairs PAX7/MyoD expression in aging skeletal muscle cells. Free Radic Biol Med 71:402–414

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.