ISSN (Print) 1225-9918
ISSN (Online) 2287-3406
Journal of Life Science **2019** Vol. 29. No. 2. 215~222 DOI : https://doi.org/10.5352/JLS.2019.29.2.215

Effect of Heat-Killed *Enterococcus faecalis*, EF-2001 on C2C12 Myoblast Damage Induced by Oxidative Stress and Muscle Volume Decreased by Sciatic Denervation in C57BL/6 Mice

Sang-Jin Chang[1], Myung-Hun Lee[1], Wan-Joong Kim[1], Yuri Chae[1], Masahiro Iwasa[1,2], Kwon-Il Han[1,2], Wan-Jae Kim[2] and Tack-Joong Kim[1,3]*

[1]*Division of Biological Science and Technology, Yonsei University, Wonju 26493, Korea*
[2]*Research & Development Center, Korea BeRM Co. Ltd., Wonju 26361, Korea*
[3]*Research & Development Center, Doctor TJ Co. Ltd., Wonju 26493, Korea*

Received December 6, 2018 / Revised December 28, 2018 / Accepted December 28, 2018

Muscle dysfunction may arise from skeletal muscle atrophy caused by aging, injury, oxidative stress, and hereditary disease. Powdered heat-killed *Enterococcus faecalis* (EF-2001) has anti-allergy, anti-inflammatory, and anti-tumor effects. However, its antioxidant and anti-atrophy effects are poorly characterized. In this study, we examined the effects of EF-2001 on muscle atrophy. To determine the protective effect of EF-2001 on oxidative stress, C2C12 myoblasts were treated with H_2O_2 to induce oxidative stress. This induced cell damage, which was reduced by treatment with EF-2001. The mechanism of EF-2001's effect was examined in response to oxidative stress. Treatment with EF-2001 reversed the expression of HSP70 and SOD1 proteins. Also, mRNA levels of *Atrogin-1/MAFbx* and *MuRF1* increased under oxidative stress conditions but decreased following EF-2001 treatment. To evaluate muscle volume, two and three dimensional models of the muscles were analyzed using micro-CT. As expected, muscle volume decreased after sciatic denervation and recovered after oral administration of EF-2001. Therefore, EF-2001 is a candidate for the treatment of muscular atrophy, and future discovery of the additional effects of EF-2001 may yield further applications as a functional food with useful activities in various fields.

Key words : EF-2001, Heat-killed *Enterococcus faecalis,* muscle atrophy, oxidative stress, sciatic denervation

Introduction

Muscle atrophy is defined as the loss of muscle tissue that occurs as a result of damage to muscle-innervating nerves or the muscle itself. The most important cause of atrophy is disuse atrophy, which is caused by the non-use of muscle. When activity decreases, muscle tone decreases, which gradually proceeds to atrophy. The physical behaviors of all the animals, including humans, are related to muscle strength, and dysfunctions of physical behavior have negative effects because they affect the ability to exercise, social life, and daily life. Muscle atrophy usually results from the reduced muscle use that occurs in an immobile state, such as during long-term care, after nerve removal, and during space travel [18].

***Corresponding author**
Tel : +82-33-760-2242, Fax : +82-33-760-2183
E-mail : ktj@yonsei.ac.kr

This is an Open-Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/3.0) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Under oxidative stress, cells activate various proteins to protect against damage and cell death. For example, superoxide dismutase 1 (SOD1) is a protein that is capable of scavenging radicals, such as intracellular reactive oxygen species ROS [9, 19, 20]. When ROS penetrates cells and cause damage, the expression of SOD1 is increased to promote homeostasis and stability. Heat shock protein 70 (HSP70) protects cells from various apoptotic stimuli, including heat shock, tumor necrosis factor, oxidative stress, ceramides, and radiation by mitigating the various effects of stress, thus allowing cells to tolerate such external stimuli, and inhibiting cell death [7, 12, 15, 16, 18, 21]. Muscle ring finger protein 1 (Murf1) is a protein that is expressed in myoblasts and contains a weak ring domain [13]. When MuRF1 expression is increased in the myoblasts, vital proteins are ubiquitinated to induce cell death [5].

The heat-killed *Enterococcus faecalis* (EF-2001) has previously been shown to have several beneficial effects on human health including anti-allergy, anti-inflammatory and anti-tumor activities [3, 4, 6], but so far anti-muscle atrophy effects of this EF-2001 have been unclear.

In the present study, we examined the effects of EF-2001 on muscle atrophy both under oxidative stress in myoblasts and following sciatic denervation in mice, and confirmed that several cellular signal expression levels, including HSP70, and SOD1 are affected by EF-2001 in situations of oxidative stress induced by disuse muscle atrophy.

Materials and Methods

Materials

The EZ-Cytox cell viability kit was purchased from Daeil Lab (Seoul, Korea). Penicillin-streptomycin and fetal bovine serum (FBS) were obtained from Capricorn (Ebsdorfergrund, Germany). N-acetyl-L-cysteine (NAC), TRI-reagent, and trypsin-EDTA solution were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phosphate buffered saline (PBS) was purchased from Gibco Life Technologies, Inc. (Rockville, MD, USA). An antibody against HSP70 was purchased from Enzo Life Sciences (AG, Switzerland). Antibodies against SOD1 and β-actin as well as anti-rabbit IgG and anti-mouse IgG were purchased from Cell Signaling Technology (Danvers, MA, USA).

Heat-killed *Enterococcus faecalis* (EF-2001)

EF-2001 is a commercially available probiotic that was originally isolated from healthy human feces. It was supplied by Nihon BeRM Co. Ltd., (Tokyo, Japan) as a heat-killed, dried powder. One gram of dried *E. faecalis* is equivalent to 7.5×10^{12} colony-forming units (CFU) prior to heat-killing.

Cell culture

C2C12 myoblasts were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich) containing 10% (v/v) FBS and penicillin-streptomycin (100 μg/ml). C2C12 myoblasts were cultured in 100 mm cell culture dishes at 37℃ in a humidified, 5% CO_2 incubator.

Cell viability

When C2C12 myoblasts reached 70% confluence, the medium was suctioned and the medium was replaced with serum-free DMEM containing EF-2001 at various concentrations (0, 25, 50, 100, 250, and 500 μg/ml) and cultured for 24 hr. Then, the C2C12 myoblasts were treated with 1 mM H_2O_2 in serum-free DMEM for 2 hr. EZ-Cytox kit reagent (10 μl) was added to each well of the plate, and then incubated for 1 h. The cell viability was measured at 450 nm with a FLx800 Microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

Evaluation of apoptotic cells

C2C12 myoblasts were seeded on a glass cover slip in a 6-well culture plate at a density of 1×10^5 cells/ml and incubated for 24 hr. The medium was replaced with DMEM with or without EF-2001 (500 μg/ml) and incubated for 24 hr. Then, the cells were treated with 1 mM H_2O_2 in serum-free DMEM for 2 hr. After incubation, the C2C12 myoblasts were fixed with 4% para-formaldehyde in PBS at room temperature for 30 min. The washed C2C12 myoblasts were mounted with mounting medium containing DAPI. The cover glass containing the fixed cells was reversed and placed on a glass slide. Sample images were acquired with a Zeiss LSM710 confocal microscope (Carl Zeiss, Oberkochen, Germany). During observation, all images were taken at the same setting**.** Images were acquired at wavelengths of 358 and 461 nm. Apoptotic cells were counted with Image J software.

Western blotting

C2C12 myoblasts were plated in 6-well culture plates at a density of 1×10^5 cells/ml and cultured in DMEM containing 10%(v/v) FBS at 37℃ for 24 hr, and the medium was replaced with serum-free DMEM with or without EF-2001 (500 μg/ml) for 24 hr. Then treated with 1 mM H_2O_2 in serum-free DMEM for 2 hr. The C2C12 myoblasts were lysed, and protein was extracted using the PRO-PREP protein extraction kit (iNtRON Biotechnology, Inc., Korea). After sonication, protein was quantified by Bradford assay (Bio-Rad, Hercules, CA, USA). Then, whole cell lysates were separated by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10~15% polyacrylamide gels. The separated proteins were transferred to polyvinylidene fluoride membrane (PVDF; Bio-Rad) using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad). The membrane was blocked overnight at 4℃ with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) and then incubated with each primary antibody (1:2,000 dilution). The primary antibodies were anti-SOD1, anti-HSP70, and anti-β-actin. After incubation with the primary antibody, the membrane was washed three times with TBS-T and then incubated for 2 hr with the secondary antibody at room temperature (1:5,000 dilution). The secondary antibody was detected by enhanced chemiluminescence (ECL) reagent, and each image was analyzed by an ImageQuant LAS 4000 system (GE Healthcare, Buckinghamshire, UK).

Real-time polymerase chain reaction

C2C12 myoblasts were seeded in a 6-well cell culture plate in DMEM containing 10%(v/v) FBS and incubated for 24 hr. After incubation, the medium was replaced with DMEM with or without EF-2001 (500 μg/ml) for 24 hr. The cells were treated with 1 mM H_2O_2 in serum-free DMEM for 2 hr. C2C12 myoblasts were lysed by using TRI-reagent (Sigma-Aldrich), and total RNA was extracted according to the manufacturer's instructions. After the RNA concentration was measured, the extracted mRNA was used as a template to synthesize cDNA. Real-time polymerase chain reaction (Real-time PCR) analysis was performed by using SYBR Green 1, a LightCycler® 96 instrument (Roche, Basel, Switzerland), and the following primers: mouse *Atrogin-1/MAFbx* sense 5′-CCATCCTCTTTCTTGCCCGT-3′ and antisense 5′-ATCACTGTCCAACCTGGCTG-3′; mouse *MuRF1* sense 5′-TGGGACAGATGAGGAGGAGG-3′ and antisense 5′-TTTACCCTCTGTGGTCACGC-3′; and mouse *GAPDH* sense 5′-AGGTCGGTGTGAACGGATTTG-3′ and antisense 5′-TGTAGACCATGTAGTTGAGGTCA-3′. The cycling conditions were as follows: 40 cycles of denaturation at 94℃ for 30 sec, annealing at 60℃ for 60 sec, and extension at 72℃ for 60 sec.

Animal experiments

Male C57BL/6 mice were purchased from Orient Bio (Gangneung, Korea). The mice were housed in wire cages and maintained under constant temperature (20-22℃) and humidity (40-50%) conditions to minimize the animals' discomfort. This animal experiment protocol was approved by the Institutional Animal Care and Use Committee (IACUC, YWC-160217-1) at Yonsei University (Wonju, Korea). The sciatic nerve in the right leg of each mouse was surgically removed to induce immobilization, except in mice in the control group. The surgery caused immobilization and muscle atrophy in the mice. Muscle atrophy was induced for 7 days. Then, EF-2001 (at 3 mg/kg and 30 mg/kg) was orally administered 10 times over 2 week period. The total experimental period was 21 days. Then, 21 days after sciatic nerve denervation, the mice were sacrificed.

Measurement of muscle volume using micro-computed tomography

Micro-Computed tomography (micro-CT) image data was obtained 14 days after denervation and before sacrifice (at 21 days after denervation) using a SkyScan 1076 micro-CT (Bruker, Germany) at a resolution of 30 μm, with the following parameters: 100 kV, 100 mA, 790 ms, and a rotation step of 1.2°. During rotation and scanning, all mice were under anesthesia. The beam-hardening errors were corrected to improve the quality of the images by flat-field correction before scanning and beam-hardening correction during reconstruction. To evaluate muscle volume, two-dimensional models and three-dimensional models of the muscles were generated by CT-Analyzer 1.11 (Bruker).

Statistical analysis

Statistical analysis of data was carried out using the SAS statistical software (SAS Institute, Cary, NC, USA). Multiple group data were analyzed using one-way analysis of variance followed by Dunnett's multiple range tests. All results are expressed as the mean ˘ standard deviation of comparative fold differences. Data are representative of three independent experiments. Significance was set at $p<0.05$.

Results

Effect of EF-2001 on the viability of C2C12 myoblasts induced by oxidative stress

Oxidative stress caused by muscle disuse and damage results in muscle atrophy. So, to determine the protective effect of EF-2001 in oxidative stress, the cell viability was analyzed by the EZ-Cytox kit. In the Fig. 1, the viability of the H_2O_2-

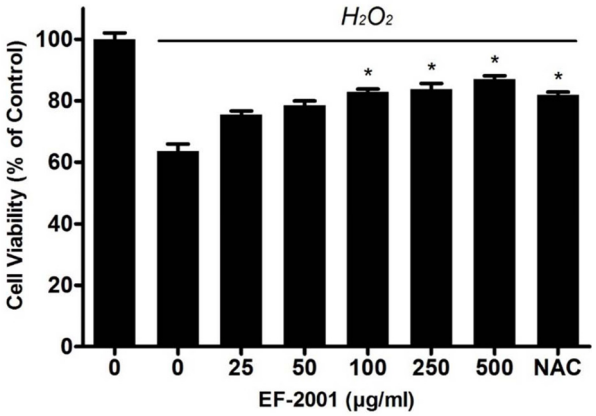

Fig. 1. Effects of Effect of EF-2001 on the viability of C2C12 myoblasts induced by oxidative stress. C2C12 myoblasts were seeded in 96-well culture plates at a density of 1×10^5 cells/ml and incubated for 24 hr. Then the cells were pretreated with EF-2001 for 24 hr. Next, the cells were pretreated with H_2O_2 in serum-free DMEM for 2 hr and washed. NAC (N-acetyl-L-cysteine; an antioxidant compound) was included as a control. Bars represent mean± SD (n=4). *$p<0.05$ indicates values that are significantly different from the EF-2001 untreated group in H_2O_2.

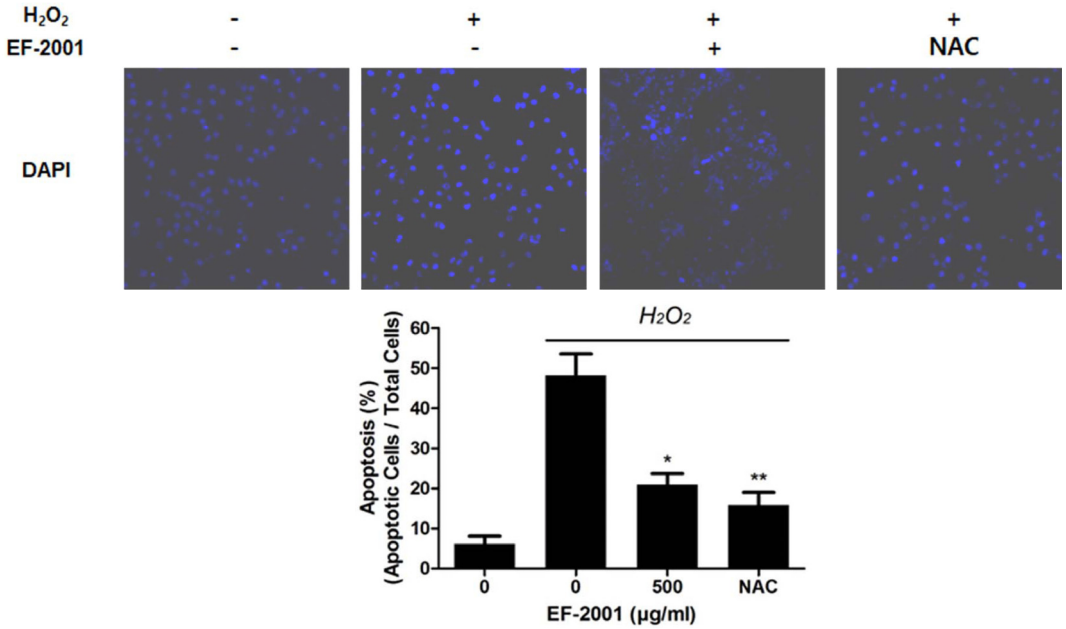

Fig. 2 Effect of EF-2001 on apoptosis in C2C12 myoblasts induced by oxidative stress. C2C12 myoblasts were cultured in 6-well culture plates and replaced with DMEM with or without EF-2001 (500 μg/ml). Next, the medium was pretreated with 1 mM H_2O_2 in DMEM. Then, the myoblasts were fixed with 4% paraformaldehyde in PBS for 30 min and mounted using mounting medium containing DAPI. Image data were collected with a confocal microscope. DAPI was detected at 358 and 461 nm. Bars represent mean ± SD (n=4). *p<0.05 indicates values that are significantly different from the EF-2001 untreated group in H_2O_2.

treated C2C12 myoblasts decreased with 63.51±2.37%. The pre-treatment of EF-2001 reduced the viability of the cells in a dose dependent manner. The viability of cells was 75.45 ±1.19, 78.46±1.46, 82.87±0.90, 83.72±1.92, and 87.05±1.08, at EF-2001 concentration of 25, 50, 100, 250, and 500 μg/ml.

Effect of EF-2001 on apoptosis in C2C12 myoblasts induced by oxidative stress

Furthermore, to verify the reduced effect of EF-2001 in the H_2O_2-treated C2C12 myoblast damage, DAPI staining was used. DAPI fluorescence was increased in H_2O_2-treated cells (48.18±5.38%) compared to that in untreated control cells (6.19±1.95%). The fluorescence in cells pre-treated with 500 μg/ml of EF-2001 was 20.99±2.73%, which is lower than that in the only H_2O_2-treated group (Fig. 2).

Effect of EF-2001 on the expression of muscle atrophy-related proteins under oxidative stress in C2C12 myoblasts

HSP70 and SOD1 show muscle-protective activity during muscle injury. To examine the mechanism of EF-2001 effect in response to oxidative stress, the expression of HSP70 and SOD1 proteins was used by Western blotting. The expression of HSP70 and SOD1 was increased by H_2O_2 treatment. However, the expression of these proteins was decreased in the presence of EF-2001 (Fig. 3).

Effect of EF-2001 on the expression of muscle atrophy-related genes under oxidative stress in C2C12 myoblasts

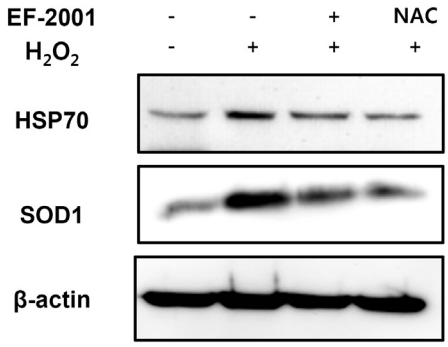

Fig. 3. Effect of EF-2001 on HSP70 and SOD1 protein expression in C2C12 myoblasts induced by oxidative stress. C2C12 myoblasts were cultured in 6-well culture plates and replaced with DMEM with or without EF-2001 (500 μg/ml). Next, the medium was pretreated with 1 mM H_2O_2 in DMEM. Then, HSP70, SOD1, and β-actin protein levels were analyzed by Western blotting.

We also examined the level of mRNAs related to sarcopenia. *MuRF1* is a ring finger protein containing a zinc finger domain that is involved in muscle cell death by ubiquitinating proteins [1, 13]. In addition, the transcriptional regulation of *Atrogin-1/MAFbx* mRNA is induced during muscle atrophy, similar to MuRF1 [14]. Therefore, we analyzed the mRNA expression of *MuRF1* and *Atrogin-1/MAFb*x by using Real-time PCR.

C2C12 myoblasts treated with H_2O_2 showed increased mRNA levels of *Atrogin-1/MAFbx* (415.16±59.12%, with levels in untreated cells set to 100%) and *MuRF1* (226.65± 43.13%, with levels in untreated cells set to 100%). However, in cells pre-treated with 500 μg/ml of EF-2001, *Atrogin-1/MAFbx* expression was decreased to 77.07±18.87%(Fig. 4A) and *MuRF1* expression was decreased to 162.18±7.96%(Fig. 4B).

Effect of EF-2001 on muscle volume caused by sciatic denervation in C57BL/6 mice

Because muscle atrophy is caused by various factors, it is difficult to test it using the human. A sciatic denervation is done with research animal models which can imitate variety of conditions that induce human skeletal muscle atrophy [2]. To induce sarcopenia in C57BL/6 mice, the sciatic nerve in the right leg of each mouse was surgically removed to induce immobilization. Sarcopenia was induced by denervation for 7 days. Then, EF-2001 was orally administered by everyday (14 days). To evaluate muscle volume, two-dimensional (2D) models and three-dimensional (3D) models of the muscles were analyzed by micro-CT (Fig. 5A). The muscle volume of denervated mice was 41.25±23.34% of that in the control mice (100%). In contrast, the mice orally administrated with EF-2001 at 3 mg/kg and 30 mg/kg reduced the decreased muscle mass in a dose-dependent manner (Fig. 5B). This result indicates that EF-2001 can restore sarcopenia caused by oxidative stress *in vitro* and *in vivo*. Thus, EF-2001 is a candidate for the treatment of sarcopenia, and future discovery of the additional effects of EF-2001 is expected to open up more possibilities as a drug or functional food with useful activities in various fields.

Discussion

We confirmed that EF-2001 prevented oxidative stress-induced cell death in muscle cells. This conclusion can be drawn from the observation that EF-2001, at 0-500 μg/ml, increased the survival of C2C12 myoblasts following induction of cell death by H_2O_2 in a dose-dependent manner (Fig. 1). Muscle atrophy is caused by cell death following muscle damage, muscle non-use, and excessive exercise. If muscle loss occurs due to sustained muscle atrophy, muscle use and movement will be limited [10, 11]. Therefore, our result suggests that EF-2001 has prevent effects in the damage of oxidative stress-induced C2C12 myoblasts. The mechanism of EF-2001 effect was examined in response to oxidative stress. SOD1, which inhibits ROS damage by scavenging ROS from cells [17], was highly expressed in H_2O_2-treated cells and was decreased following EF-2001 treatment. HSP70 is a heat shock protein that prevents cells from dying in the presence of external stress [8]. HSP70 was also expressed

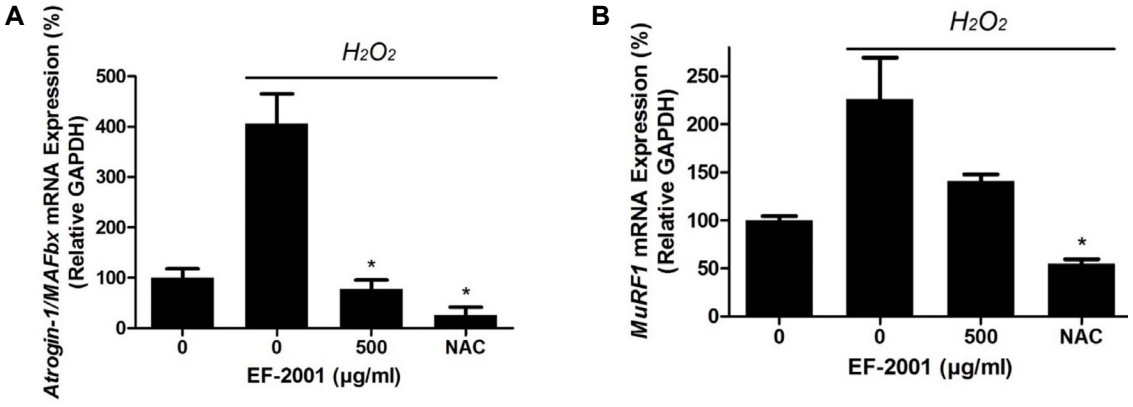

Fig. 4. Effect of EF-2001 on *Atrogin-1/MAFbx* and *MuRF1* mRNA expression in C2C12 myoblasts induced by oxidative stress. C2C12 myoblasts were cultured in 6-well culture plates and replaced with DMEM with or without EF-2001 (500 μg/ml). Next, the medium was pretreated with 1 mM H_2O_2 in DMEM. Then, the mRNA expressions of *Atrogin-1/MAFbx* and *MuRF1* were analyzed by real-time PCR. Bars represent mean ± SD (n=4). *p<0.05 indicates values that are significantly different from the EF-2001 untreated group in H_2O_2.

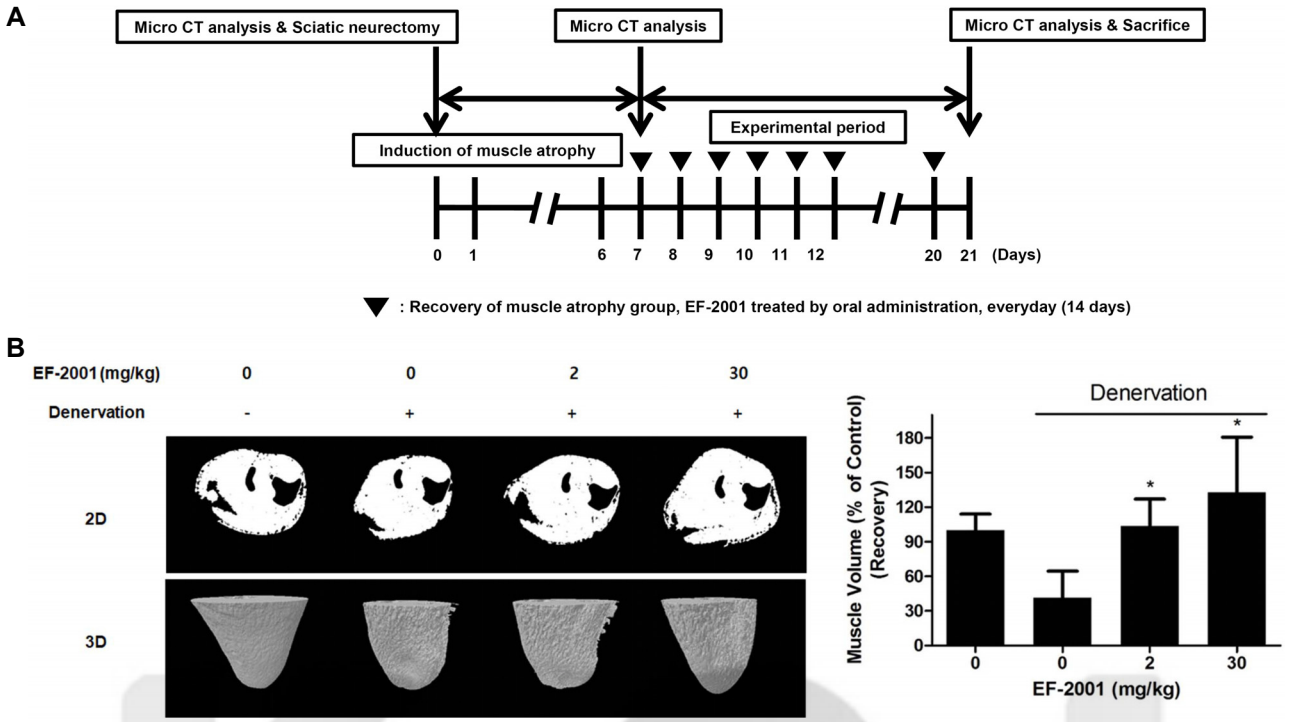

Fig. 5. Effect of EF-2001 on muscle atrophy induced by sciatic denervation in C57BL/6 mice. (A) Male C57BL/6 mice were used after acclimation to the environment. Three CT scans were performed: before the sciatic neurectomy, 7 days after atrophy induction, and before sacrifice (at 21 days). (B) Muscle volume in the mice was assessed by micro-CT at various time points before after sciatic denervation with and without EF-2001 treatment. Bars represent mean ± SD (n=6). *p<0.05 indicates values that are significantly different from the EF-2001 untreated group after sciatic denervation.

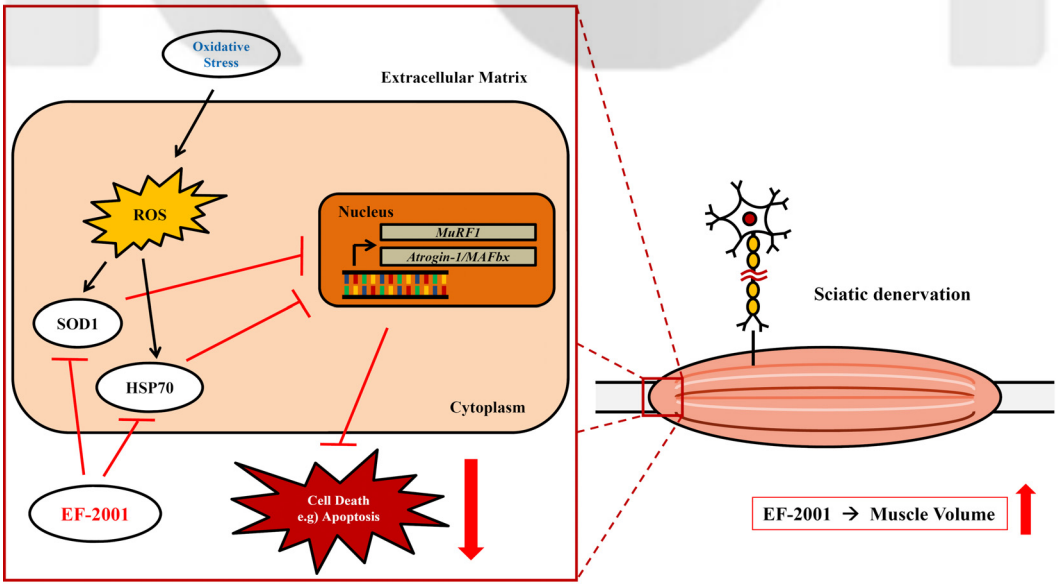

Fig. 6. EF-2001 can restore muscle atrophy caused by oxidative stress *in vitro* and *in vivo*.

in H_2O_2-treated cells and was decreased following treatment with EF-2001. Based on these protein expression results, we examined the expression of mRNAs related to muscle atrophy. The mRNA levels of *Atrogin-1/MAFbx* and *MuRF1* increased under oxidative stress conditions, but decreased following EF-2001 treatment. Our results suggest that EF-2001 inhibits the muscle injury-related protein expression, and the mRNA level of *Atrogin-1/MAFbx* and *MuRF1* in-

duced by oxidative stress in C2C12 myoblasts. Based on these *in vitro* data, we next evaluated the effects of EF-2001 on muscle recovery *in vivo* by inducing muscle atrophy in C57BL/6 mice. Muscle volume, as 2D and 3D models of the muscle atrophy, decreased after sciatic denervation, and this decrease was recovered by administration of EF-2001. Previous studies have shown that powdered heat-killed *Enterococcus faecalis* has anti-allergy, anti-inflammatory and anti-tumor effects [3, 4, 6]. This study confirms that heat-killed *Enterococcus faecalis* can restore muscle atrophy caused by oxidative stress *in vitro* and *in vivo*. Thus, heat-killed *Enterococcus faecalis* is a candidate for the treatment of muscular atrophy, and future discovery of the additional effects of heat-killed *Enterococcus faecalis* is expected to open up more possibilities as a functional food with useful activities in various fields.

Acknowledgements

This research was financially supported by the Ministry of SMEs and Startups (MSS), Korea, under the "Regional Specialized Industry Development Program (R&D, R0006434)" supervised by the Korea Institute for Advancement of Technology (KIAT).

References

1. Berridge, M. V., Herst, P. M. and Tan, A. S. 2005. Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. *Biotechnol. Annu. Rev.* **11**, 127-152.
2. Booth, F. W. 1982. Effect of limb immobilization on skeletal muscle. *J. Appl. Physiol. Respir. Environ. Exerc. Physiol.* **52**, 1113-1118.
3. Choi, E. J., Iwasa, M., Han, K. I., Kim, W. J., Tang, Y., Han, W. C., Kim, E. K. and Park, Z. Y. 2016. Effect of *Enterococcus faecalis* EF-2001 on experimentally induced atopic eczema in mice. *Food Sci. Biotechnol.* **25**, 1087-1093.
4. Choi, M. S., Chang, S. J., Chae, Y., Lee, M. H., Kim, W. J., Iwasa, M., Han, K. I., Kim, W. J. and Kim, T. J. 2018. Anti-inflammatory Effect of Heat-Killed Enterococcus faecalis EF-2001. *J. Life Sci.* **28,** 1361-136.
5. Frost, R. A., Nystrom, G. J., Jefferson, L. S. and Lang, C. H. 2007. Hormone, cytokine, and nutritional regulation of sepsis-induced increases in atrogin-1 and MuRF1 in skeletal muscle. *Am. J. Physiol. Endocrinol. Metab.* **292**, E501-512.
6. Gu, Y. H., Choi, H., Yamashita, T., Kang, K. M., Iwasa, M., Lee, M. J., Lee, K. H. and Kim, C. H. 2017. Pharmaceutical Production of Anti-tumor and Immune-potentiating *Enterococcus faecalis*-2001 β-glucans: Enhanced Activity of Macrophage and Lymphocytes in Tumor-implanted Mice. *Curr. Pharm. Biotechnol.* **18**, 653-661.
7. Haycock, J. W., MacNeil, S., Jones, P., Harris, J. B. and Mantle, D. 1996. Oxidative damage to muscle protein in Duchenne muscular dystrophy. *Neuroreport* **8**, 357-361.
8. Jäättelä, M., Wissing, D., Bauer, P. A. and Li, G. C. 1992. Major heat shock protein hsp70 protects tumor cells from tumor necrosis factor cytotoxicity. *EMBO J.* **11**, 3507-3512.
9. Jackson, M. J. and Farrell, S. O. 1993. Free radicals and muscle damage. *Br. Med. Bull.* **49**, 630-641.
10. Lee, Y. H., Kim, W. J., Lee, M. H., Kim. S. Y., Seo, D. H., Kim. H. S., Gelinsky, M. and Kim, T. J. 2016. Anti-skeletal muscle atrophy effect of *Oenothera odorata* root extract via reactive oxygen species-dependent signaling pathways in cellular and mouse model. *Biosci. Biotechnol. Biochem.* **80,** 80-88.
11. Lee, Y. H., Seo, D. H., Park, J. H., Kabayama, K., Opitz, J., Lee, K. H., Kim, H. S. and Kim, T. J. 2015. Effect of Oenothera odorata root extract on microgravity and disuse-induced muscle atrophy. *Evid. Based Complement. Alternat. Med.* **2015,** 130513.
12. Li, C. Y., Lee, J. S., Ko, Y. G., Kim, J. I. and Seo, J. S. 2000. Heat shock protein 70 inhibits apoptosis down stream of cytochrome c release and upstream of caspase-3 activation. *J. Biol. Chem.* **275**, 25665-25671.
13. Mrosek, M., Meier, S., Ucurum-Fotiadis, Z., von Castelmur, E., Hedbom, E., Lustig, A., Grzesiek, S., Labeit, D., Labeit, S. and Mayans, O. 2008. Structural analysis of B-Box 2 from MuRF1: identification of a novel self-association pattern in a RING-like fold. *Biochemistry* **47**, 10722-10730.
14. Natanek, S. A., Riddoch-Contreras, J., Marsh, G. S., Hopkinson, N. S., Moxham, J., Man, W. D., Kemp, P. R. and Polkey, M. I. 2013. MuRF-1 and atrogin-1 protein expression and quadriceps fiber size and muscle mass in stable patients with COPD. *COPD* **10**, 618-624.
15. Ogata, T., Oishi, Y., Higashida, K., Higuchi, M. and Muraoka, I. 2009. Prolonged exercise training induces long-term enhancement of HSP70 expression in rat plantaris muscle. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **296**, R1557-1563.
16. Oksala, N. K., Ekmekçi, F. G., Özsoy, E., Kirankaya, Ş., Kokkola, T., Emecen, G., Lappalainen, J., Kaarniranta, K. and Atalay, M. 2014. Natural thermal adaptation increases heat shock protein levels and decreases oxidative stress. *Redox Biol.* **3**, 25-28.
17. Powers, S. K. 2014. Can antioxidants protect against disuse muscle atrophy? *Sports Med.* **44**, S155-165.
18. Rodriguez, M. C. and Tarnopolsky, M. A. 2003. Patients with dystrophinopathy show evidence of increased oxidative stress. *Free Radic. Biol. Med.* **34,** 1217-1220.
19. Sugawara, T., Lewén, A., Gasche, Y., Yu, F. and Chan, P. H. 2002. Overexpression of SOD1 protects vulnerable motor neurons after spinal cord injury by attenuating mitochondrial cytochrome c release. *FASEB J.* **16,** 1997-1999.
20. Tsang, C. K., Liu, Y., Thomas, J., Zhang, Y. and Zheng, X. S. 2014. Superoxide dismutase 1 acts as a nuclear transcription factor to regulate oxidative stress resistance. *Nat. Commun.* **5**, 3446.
21. Zylicz, M. and Wawrzynow, A. 2001. Insights into the function of Hsp70 chaperones. *IUBMB Life* **51**, 283-287.