

## Research Paper

# Microcurrent Electrical Nerve Stimulation Facilitates Regrowth of Mouse Soleus Muscle

Yoshitaka Ohno<sup>1</sup>, Hiroto Fujiya<sup>2</sup>, Ayumi Goto<sup>3</sup>, Ayane Nakamura<sup>1</sup>, Yuka Nishiura<sup>1</sup>, Takao Sugiura<sup>4</sup>, Yoshinobu Ohira<sup>5</sup>, Toshitada Yoshioka<sup>6</sup>, Katsumasa Goto<sup>1,3</sup> ✉

1. Laboratory of Physiology, School of Health Sciences, Toyohashi SOZO University, Toyohashi, Aichi, Japan;
2. Department of Sports Medicine, St. Marianna University School of Medicine, Kawasaki, Kanagawa, Japan;
3. Department of Physiology, Graduate School of Health Sciences, Toyohashi SOZO University, Toyohashi, Aichi, Japan;
4. Faculty of Education, Yamaguchi University, Yamaguchi-City, Yamaguchi, Japan;
5. Graduate School of Medicine, Osaka University, Toyonaka, Osaka, Japan;
6. Hirosaki Gakuin University, Hirosaki, Aomori, Japan.

✉ Corresponding author: Katsumasa Goto, Ph.D. Department of Physiology, Graduate School of Health Sciences, Toyohashi SOZO University, 20-1 Matsushita, Ushikawa, Toyohashi, Aichi 440-8511, Japan. TEL +81-50-2017-2272 FAX +81-532-55-0803 E-mail: gotok@sepia.ocn.ne.jp.

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## Abstract

Microcurrent electrical nerve stimulation (MENS) has been used to facilitate recovery from skeletal muscle injury. However, the effects of MENS on unloading-associated atrophied skeletal muscle remain unclear. Effects of MENS on the regrowing process of unloading-associated atrophied skeletal muscle were investigated. Male C57BL/6J mice (10-week old) were randomly assigned to untreated normal recovery (C) and MENS-treated (M) groups. Mice of both groups are subjected to continuous hindlimb suspension (HS) for 2 weeks followed by 7 days of ambulation recovery. Mice in M group were treated with MENS for 60 min 1, 3, and 5 days following HS, respectively, under anesthesia. The intensity, the frequency, and the pulse width of MENS were set at 10  $\mu$ A, 0.3 Hz, and 250 msec, respectively. Soleus muscles were dissected before and immediately after, 1, 3 and 7 days after HS. Soleus muscle wet weight and protein content were decreased by HS. The regrowth of atrophied soleus muscle in M group was faster than that in C group. Decrease in the reloading-induced necrosis of atrophied soleus was facilitated by MENS. Significant increases in phosphorylated levels of p70 S6 kinase and protein kinase B (Akt) in M group were observed, compared with C group. These observations are consistent with that MENS facilitated regrowth of atrophied soleus muscle. MENS may be a potential extracellular stimulus to activate the intracellular signals involved in protein synthesis.

Key words: skeletal muscle; atrophy; regrowth; microcurrent electrical nerve stimulation; intracellular signal; rehabilitation.

## Introduction

Skeletal muscle injuries are common sports trauma. Not only professional but also recreational athletes having skeletal muscle injuries are forced to be inactive for a certain period. Muscle injuries-associated inactivity is one of major causes of skeletal muscle atrophy and weakness.<sup>1-3</sup> Skeletal muscle atrophy is another serious problem for the

recovery from sport injury. Various efforts have been made to establish effective rehabilitation therapy for the facilitation of recovery of injured and atrophied skeletal muscle.

Microcurrent electrical nerve stimulation (MENS) was developed as a physical therapy modality delivering current in the microampere range.

MENS has a reducing effect of signs and symptoms of muscle damage.<sup>4,5</sup> However, molecular mechanism for MENS-associated stimulation of the regenerative potential of injured skeletal muscle is still unclear because the regeneration of injured skeletal muscle is a complicated process. Because increase in protein synthesis is essential during the regeneration of injured<sup>6-9</sup> as well as atrophied<sup>10</sup> skeletal muscle, it is strongly suggested that MENS may facilitate the recovery of atrophied skeletal muscle via stimulation of protein synthesis. However, there is no report regarding the effects of MENS on the regrowth of atrophied skeletal muscle.

Akt, also known as protein kinase B (PKB), is a serine/threonine-specific protein kinase that plays a key role in multiple cellular processes such as protein synthesis, glucose metabolism, apoptosis, cell proliferation, and other cell functions.<sup>11-14</sup> It is generally accepted that not only Akt but also p70 S6 kinase (p70S6K) plays a key role in integrating intracellular signaling in protein synthesis of skeletal muscle cells.<sup>10,15-17</sup> Phosphorylated form of both molecules are enzymatically active.<sup>12,14,18,19</sup> Akt has been shown to be activated by mechanical stretch that could induce muscle hypertrophy.<sup>20</sup> Akt is also known to regulate p70S6K indirectly via activation of the mTOR pathway.<sup>21</sup> Up-regulation of phosphorylated p70S6K (p-p70S6K) has been implicated in elevated muscle protein synthesis by increasing mRNA translation through phosphorylation of ribosomal protein components.<sup>10</sup> Increase in phosphorylated forms of Akt (p-Akt) and p-p70S6K was observed during muscle hypertrophy<sup>22</sup> and regrowth of atrophied skeletal muscle.<sup>23</sup> However, there is no evidence regarding the effects of MENS on these intracellular signals associated with muscle protein synthesis.

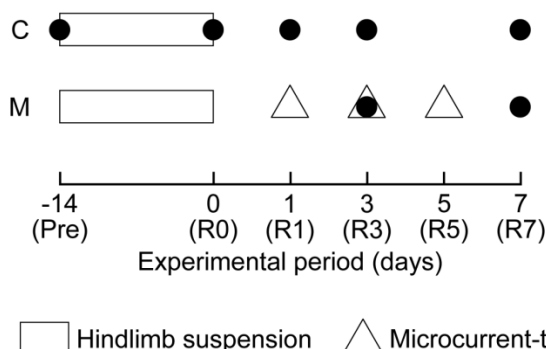
The purpose of the present study was to investigate the effects of MENS on the regrowth of atrophied skeletal muscle. Skeletal muscle atrophy with the loss of muscle mass and strength results from muscle unloading, which is associated with space-

flight<sup>24</sup> and bed rest.<sup>25</sup> It has been generally accepted that reduced generation of active and passive tensions<sup>26,27</sup> in microgravitational environment most likely contributes to muscle wasting process.<sup>28</sup> Ground-based hindlimb unloading rodent model has been widely used for space flight-induced muscle atrophy. Numerous studies have clearly shown that 7 to 14 days of hindlimb suspension (HS) of rodents causes atrophy of hindlimb skeletal muscle, especially antigravitational soleus muscle. In addition, it has been reported that inflammation, membrane lysis, and necrosis in reloading on unloading-associated atrophied rat soleus muscle were observed.<sup>29</sup> On the other hand, unloading-associated atrophied soleus muscles of rats and mice are grown by reloading and muscle mass recovers to the control level.<sup>10,30,31</sup> Therefore, in the present study, we investigate the effects of MENS on regeneration of muscle necrosis and regrowth of atrophied soleus muscle by using hindlimb unloading rodent model. We also discussed about the effect of MENS on the intracellular signals associated with protein synthesis during regrowth of atrophied soleus muscle. Evidences from this study showed that MENS facilitated regrowth of atrophied skeletal muscle via the stimulation of muscle protein synthesis.

## Materials and Method

### Animals and Grouping

All experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health (Bethesda, MD, USA) and were approved by the Animal Use Committee of Toyohashi SOZO University. Male C57BL/6J mice, aged 10 weeks old were used (n = 35). Mice were randomly divided into two groups: (i) untreated normal recovery (C, n = 25) and (ii) MENS-treated (M, n = 10) groups (Fig. 1).



● Sampling □ Hindlimb suspension △ Microcurrent-treatment

**Fig 1.** Summary of the experimental protocol. C: untreated control group; M: microcurrent electrical nerve stimulation (MENS)-treated group. Pre: before hindlimb suspension; R0, R1, R3, R5, and R7: immediately after (0), 1, 3, 5, and 7 days after the hindlimb suspension.

Five mice of C group were assigned as the cage control and killed immediately before the initiation of HS. To induce unloading-associated muscle atrophy, all mice in both groups, except the cage control, were subjected to HS for 2 weeks. The HS was performed following the methods described previously.<sup>32,33</sup> Briefly, tails of the HS mice were cleaned, and were loosely surrounded by adhesive tapes cross-sectionally, fixing a string at the dorsal side of the tail, to maintain the blood flow intact. The string was fastened to the roof of the cage at a height allowing the forelimbs to support the weight, yet preventing the hindlimbs from touching the floor and the sides of the cage (20 x 31 cm and 13.5 cm height). The mice could reach food and water freely by using their forelimbs. Five mice of both groups, except for the period of HS, were housed in a home cage with the same size as for HS in a clean room controlled at approximately 23°C and at 55% humidity with a 12/12 hours light-dark cycle. Solid diet and water were provided *ad libitum*.

### MENS treatment

Immediately after HS, ambulation recovery was performed on the mice in both groups. Following 1, 3 and 5 days after HS, the both hindlimbs of mice in M group were treated with MENS (intensity: 10  $\mu$ A, frequency: 0.3 Hz, pulse width: 250 msec) by using an electrical stimulator (Trio300, Ito Co., Ltd., Tokyo, Japan) for 60 min under anesthesia with *i.p.* injection of sodium pentobarbital (50 mg/kg). Mice in C group were also anesthetized for 60 min without MENS treatment. Epilation of mouse hindlimbs was performed by using a commercial hair remover for human. Two electrodes were placed on the distal posterior side of the knee joint and the proximal posterior side of ankle joint, respectively.<sup>34</sup> The condition for MENS was determined considering the total current for animals, compared with the studies by using human<sup>35</sup> and rats.<sup>36</sup> In the present study, no muscle contraction in hindlimbs of mice was observed during MENS.

### Sampling

Soleus muscles in C group were dissected from both hindlimbs before (Pre) and immediately after (R0), 1 (R1), 3 (R3), and 7 (R7) days after HS (n = 5 in each day). Soleus in M group were also dissected at R3 and R7 (n = 5 in each day). Previous study showed that reloading-associated necrotic muscle fibers were observed 2<sup>nd</sup> and 4<sup>th</sup> days of reloading on atrophied soleus muscle that was induced by 10 days of HS.<sup>37</sup> In addition, immediately after HS and 7<sup>th</sup> day of reloading, necrotic area in soleus muscle was similar to untreated control.<sup>37</sup> Regenerating fibers of soleus muscle

were clearly observed 2<sup>nd</sup> and 4<sup>th</sup> days of reloading following HS.<sup>38</sup> Unloading-associated atrophied soleus muscle mass is increased by reloading and the muscle weight recovers to the control levels 7<sup>th</sup> day of reloading following 10 days of HS.<sup>39</sup> Judging from these evidences, we examined the effects of MENS on regeneration and regrowth of unloading-associated atrophied soleus muscle at R3 and R7.

After the experimental period, the animals of each group were sacrificed by cervical dislocation. Both soleus muscles were excised from both hindlimbs. Soleus was trimmed of excess fat and connective tissues, weighed, frozen in liquid nitrogen, and stored at -80°C until analyses.

### Muscle Protein Content

Left soleus muscles were cross-sectionally cut into halves at the midbelly region. Half of the muscles were used for the measurement of muscular protein content. The muscles were homogenized in 0.4 ml of tissue lysis reagent (CellLytic™-MT, Sigma, St. Louis, MO, USA) and completely solubilized by alkaline treatment with 2 N NaOH at 37°C for 1 h. Protein content of the tissue lysate was determined by using the Bradford technique (protein assay kit; Bio-Rad, Hercules, CA, USA) and bovine serum albumin (Sigma) as the standard. Total protein content in whole muscle was then calculated.

### Western Blotting and Densitometry

The remaining half of the left soleus muscles were homogenized in 0.4 ml isolation buffer of tissue lysis reagent (CellLytic™-MT, Sigma) with 10% (v/v) Protease Inhibitor Cocktail (P8340, Sigma) and 1% (v/v) Phosphatase Inhibitor Cocktail (524625, Calbiochem, San Diego, CA, USA) and centrifuged at 12,000 rpm (4°C for 10 min), then the supernatant was collected. The supernatant was mixed with sodium-dodecylsulfate (SDS) sample buffer: 30% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2.3% (w/v) SDS, 62.5 mM Tris-HCl, 0.05% (w/v) bromophenol blue and pH 6.8, at a concentration of 1 mg protein/ml and was boiled for 3 min. The SDS-polyacrylamide gel electrophoresis (PAGE) was carried out on 8 or 12% polyacrylamide containing 0.5% SDS at a constant current of 20 mA for 120 min. Equal amounts of protein (20  $\mu$ g) were loaded on each gel. Molecular weight markers (Bio-Rad Precision Markers) were applied to both sides of 14 lanes as the internal controls for transfer process or electrophoresis.

Following SDS-PAGE, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (0.2  $\mu$ m pore size, Bio-Rad) using a Bio-Rad mini trans-blot cell at a constant voltage of 100 V for 60 min

at 4°C. After the transfer, the membranes were blocked for 1 h using a blocking buffer (5% skim milk with 0.1% Tween 20 in Tris-buffered saline with pH 7.5). Then, the membranes were incubated for 2 h with a polyclonal antibody (p-Akt, 9271, Cell Signaling Technology Inc. (CST), Beverly, Mass., USA; Akt, 9272, CST; p-p70S6K, 9205, CST; p70S6K, 9202, CST) and then reacted with a secondary antibody (goat anti-rabbit immunoglobulin G conjugate to horseradish peroxidase; CST) for 2 h. After the final wash, protein bands were visualized using chemiluminescence (ECL Advance Western blotting kit; GE Healthcare, Little Chalfont, UK), and signal density was measured by using Light-Capture (AE-6971) with CS Analyzer version 2.08b (ATTO Corporation, Tokyo, Japan). In the present study, the activation levels of Akt and p70S6K were evaluated by using the expression levels of the phosphorylated forms relative to total expression forms (phosphorylated and non-phosphorylated forms). Each sample was investigated in duplicate, at least, to ensure that results were not influenced by loading errors.

### Histochemical Analyses

Frozen right soleus muscles were cut cross-sectionally into halves. Serial transverse cryosections (8  $\mu$ m thick) of the midbelly region of the proximal side were sliced at -20°C and mounted on glass slides. The slides were air dried, fixed in citrate/acetone solution for 30 seconds at room temperature, and stained to analyze the necrotic area by using acid phosphatase staining. Histochemical staining of acid phosphatase reaction is generally used to evaluate muscle cell necrosis.<sup>40</sup> The images of muscle sections in both stainings were incorporated into a personal computer (DP-BSW Ver.02.02, Olympus, Tokyo, Japan) by using a microscope (IX81 with DP70, Olympus). In acid phosphatase staining, the necrotic area and whole cross-sectional area of soleus muscle, respectively, were measured using the National Institutes of Health Image J 1.38X (NIH, Bethesda, MD, USA) software for Windows and then the percentage of necrotic area relative to the whole cross-sectional area of soleus was calculated.

### Statistical Analysis

All values were expressed as means  $\pm$  SEM. Statistical significance for changes of the measurements among Pre, R0, and R1 in C group was analyzed by using one-way analysis of variance (ANOVA) followed by the Scheffe's F post hoc test. Statistical significance for the changes of measurements at R3 and R7 in both groups was analyzed by using two-way (treatment  $\times$  time) ANOVA followed by Scheffe's F post hoc test. When a significant interaction between

two main effects (treatment and time) was observed, one-way ANOVA followed by Scheffe's F post hoc test was performed. The significance level was accepted at  $p < 0.05$ .

### Results

In the present study, body weight was significantly decreased by 2 weeks of HS (Fig. 2A,  $p < 0.05$ ). The weight showed a trend to recover to the initial level (Pre) during the reloading period. Body weight at R3 in M group was lower than that in C group ( $p < 0.05$ ). However, there was no significant difference in body weight between C and M groups at R7. Muscle wet weight and protein content of soleus were also significantly decreased by HS (Figs. 2B-E,  $p < 0.05$ ). Then, a gradual and significant increase in muscle wet weight and protein content of soleus in both C and M groups was also observed during the reloading period. There was a significant difference in relative wet weight and protein content between R3 and R7 (Figs. 2D and E,  $p < 0.05$ ). Relative wet weight and protein content in M group were significantly higher than those in C group during the reloading ( $p < 0.05$ ).

Necrotic region was observed in reloaded soleus muscle after HS (Fig. 3). The percentage of necrotic area relative to the whole cross-sectional area at R1 was approximately  $3.1 \pm 0.3\%$ , although no necrotic area was observed at Pre and R0. The percentage of necrotic area of soleus muscle was gradually decreased during the recovery period. There is a significant difference in the percentage of necrotic area between R3 and R7 (Fig. 3B,  $p < 0.05$ ). The percentage of necrotic area in M group was significantly lower than that in C group during the regrowing phase ( $p < 0.05$ ).

In the present study, the expression levels of Akt and p70S6K in response to 2 weeks of HS followed by 7 days of reloading were investigated by using Western blotting analyses (Fig. 4). There were no significant changes in the mean expression level of Akt at R0 and R1 (Fig. 4B). There is no difference in the expression level of p-Akt between C and M groups at R3. However, the expression of p-Akt in M group was significantly increased within 7 days of reloading ( $p < 0.05$ ). There were significant differences in the expression level of p-Akt between C and M group at R7 ( $p < 0.05$ ), and between R3 and R7 in M group ( $p < 0.05$ ).

There was also no significant change in the mean expression level of p70S6K at R0 (Fig. 4C). Relative expression level of p-p70S6K at R1 was significantly higher than that at Pre and R0 ( $p < 0.05$ ). The expression levels of p-p70S6K in M groups were significantly higher than those in C group during 3<sup>rd</sup> and 7<sup>th</sup> days of reloading ( $p < 0.05$ ).

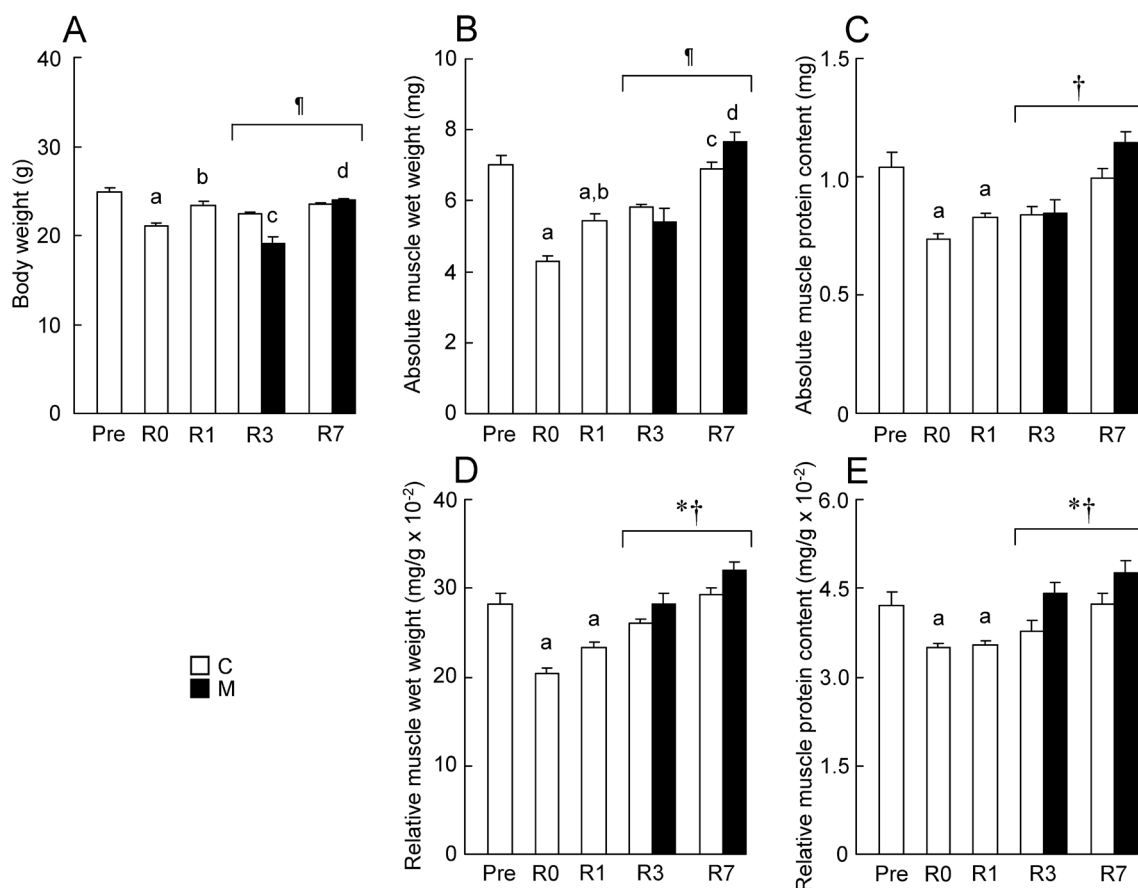
## Discussion

The present study demonstrated that the application of MENS facilitated regrowth of atrophied soleus muscle induced by hindlimb unloading. This is the first report concerning the effects of MENS on regrowth of atrophied skeletal muscle. MENS also promoted a decrease of the area of necrosis which was induced by reloading following HS. The levels of p-p70S6K (3<sup>rd</sup> and 7<sup>th</sup> day of reloading) and p-Akt (7<sup>th</sup> day of reloading) were up-regulated by MENS.

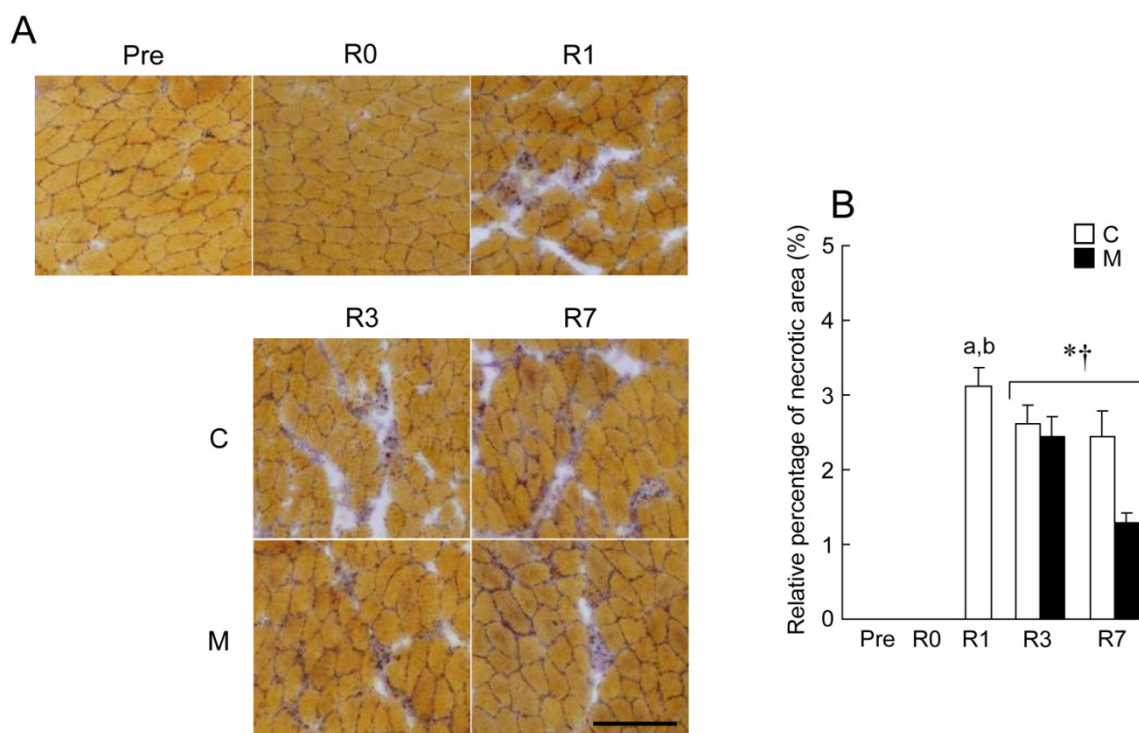
In the present study, losses of body weight, wet weight and protein content of soleus muscle were observed following 2 weeks of HS. These results were consistent with previously reported data in rat<sup>41</sup> and mice.<sup>33</sup> Decreased body weight showed a trend to recover to the initial level (Pre) during the reloading period. However, a significant decrease of body weight in MENS-treated M group was observed at R3,

but not in C group. Thereafter, the weight recovered to control levels (C group) within 7 days of reloading. In the present study, MENS-induced muscle contraction was not observed. In addition, there is no report showing harmful effects of MENS on the skeletal muscle. A transient decrease of body weight in M group might be attributed to an immediately early response to MENS treatment.

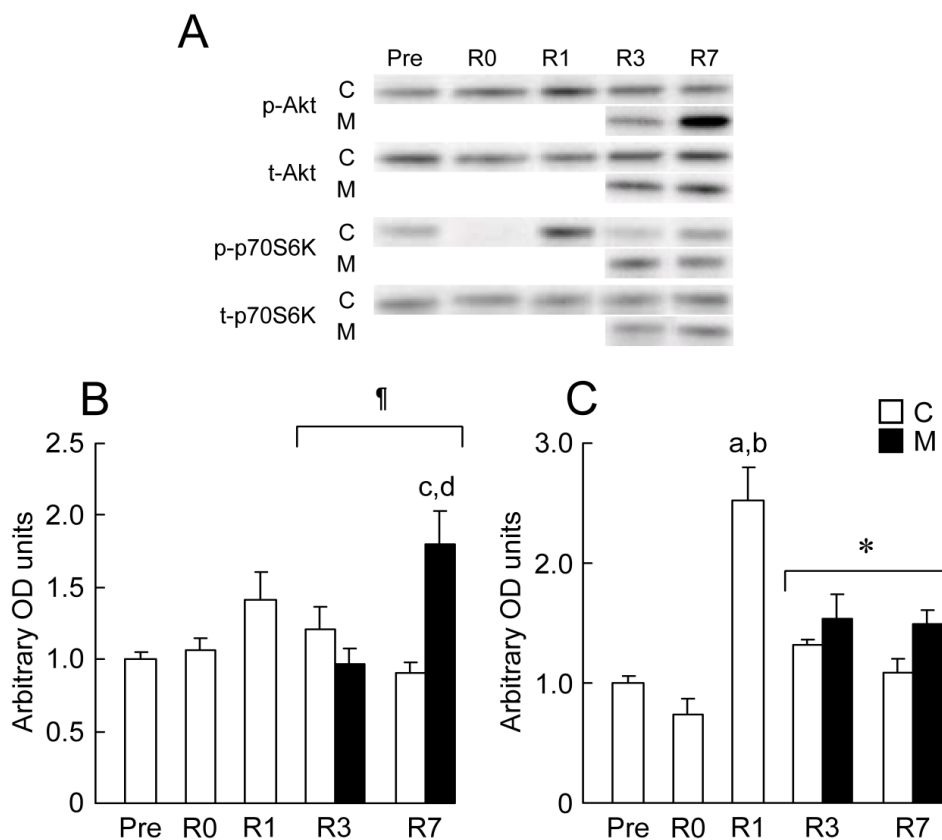
We also found that muscle mass in C group was gradually increased by reloading following HS (Fig. 2). In addition, MENS facilitated the recovery of muscle mass during the regrowing phase of unloading-induced atrophied muscle. Reloading on unloading-induced atrophied skeletal muscle stimulates protein synthesis in the muscle, and recovers muscle mass.<sup>42</sup> MENS may enhance the stimulating effects of reloading on protein synthesis of skeletal muscles during regrowth of unloading-associated atrophied muscle.



**Fig 2.** Effects of MENS on the body weight (A), absolute muscle wet weight (B), absolute muscle protein content (C), muscle wet weight relative to body weight (D), muscle protein content relative to body weight (E) of soleus. See figure 1 for abbreviations. Values are means  $\pm$  SEM. n = 5 per group each day. \*, †, and ‡: Significant main effect of treatment and time, and interaction, respectively, p < 0.05. a, b, c, and d: Significant different from Pre, R0, R3 in C group and R3 in M group, respectively, p < 0.05.



**Fig 3.** Effects of MENS on reloading-associated necrosis of soleus muscle. A: typical images of transverse cryosections of the midbelly region of soleus muscle stained with acid phosphatase. Scale bars = 100  $\mu$ m. There was no necrosis before (Pre) and immediately after (R0) hindlimb suspension. B: the percentage of necrotic area relative to the whole cross-sectional area of soleus. See figure 1 for abbreviations. Values are means  $\pm$  SEM. n = 5 per group each day. \* and †: Significant main effect of treatment and time, respectively,  $p < 0.05$ . a and b: Significant different from Pre and R0 in C group, respectively,  $p < 0.05$ .



**Fig 4.** Representative expression patterns (A) and the mean expression levels of phosphorylated protein kinase B (Akt)/total Akt (B), phosphorylated p70 S6 kinase (p70S6K)/total p70S6K (C) in response to MENS. p-Akt: phosphorylated Akt; t-Akt: total Akt; p-p70S6K: phosphorylated p70S6K; t-p70S6K: total p70S6K. See figure 1 for other abbreviations. Values are means  $\pm$  SEM. n = 5 per group each day. \* and †: Significant main effect of treatment and interaction, respectively,  $p < 0.05$ . a, b, c, and d: Significant different from Pre, R0, R7 in C group and R3 in M group, respectively,  $p < 0.05$ .

As unloading-induced skeletal muscle atrophy results in muscle weakness, muscle inflammation, muscle membrane lysis and necrosis occur in reloaded skeletal muscle after unloading.<sup>29</sup> In the present study, necrotic region in soleus muscle was also observed at R1. Thereafter, the necrotic area was gradually decreased during the reloading period. In addition, the application of MENS facilitated a decrease in the necrotic area (Fig 3). MENS may have a facilitating effect on the regeneration of injured skeletal muscle.

In the present study, no significant changes in the mean expression levels of p-Akt and p-p70S6K in soleus muscle were observed immediately after HS. There are controversial evidences regarding the responses of relative expression level of p-Akt or Akt activity to HS. Relative expression level of p-Akt in mice soleus<sup>43</sup> and activity of Akt in rat soleus<sup>44</sup> did not change following 7-14 days of HS. These observations were consistent with the results of the present study. On the other hand, there is other report that showed a decrease in relative expression of p-Akt in rat soleus muscle following 10 days of HS.<sup>10</sup> It is still unclear that physiological function of p-Akt during unloading-associated muscle atrophy.

In the present study, the expression level of p-p70S6K in soleus muscle increased during the early recovery period (i.e., R1). The previous study showed the elevated level of phosphorylated ribosomal protein S6, which is a downstream of p70S6K, persisted several days following the increase in p-p70S6K in rat soleus muscle, resulting that muscle protein synthesis was facilitated during recovery from muscle atrophy.<sup>10</sup> Reloading on unloading-induced atrophied skeletal muscle may stimulate protein synthesis via activation of p70S6K. In this study, we found that Akt-independent activation of p70S6K was induced by 1 day of reloading following unloading. Although Akt regulates the activation level of p70S6K via the mTOR pathway,<sup>21</sup> Akt-independent regulation of p-p70S6K has been suggested observed by reloading on rat soleus<sup>10</sup> and tibialis anterior muscles<sup>45</sup> following hindlimb unloading.<sup>10,46</sup> It has been suggested that p70S6K activation by mechanical stimulation on skeletal muscle is Akt-independent.<sup>46</sup> Akt-independent activation of p70S6K in skeletal muscle may be induced by mechanical stress during reloading following unloading.

We also found that Akt-independent activation of p70S6K was induced by MENS, compared with untreated control group (i.e., R3). In addition, MENS-associated activations of both Akt and p70S6K were also observed at R7. There was no evidence regarding the effects of MENS as well as a traditional electrical stimulation, which could induce muscle

contraction, on Akt/p70S6K signaling pathway in the regrowing phase of atrophied skeletal muscle following hindlimb unloading. It has been reported that HS-associated muscle atrophy of rat soleus could not be prevented by a traditional electrical stimulation even though the stimulation induced Akt-independent activation of p70S6K.<sup>45</sup> Molecular mechanism for MENS-associated Akt-independent activation of p70S6K is still unclear. Collectively, all the aforementioned results suggested that MENS might have additional stimulating effects on muscle protein synthesis during the reloading period via activation of p70S6K, resulting that the recovery of atrophied skeletal muscle might be facilitated by MENS treatment. On the other hand, we have no clear explanation for Akt activation induced by MENS treatment. It is also still unclear whether MENS treatment could prevent or attenuate unloading-associated muscle atrophy.

It has been reported that electrical stimulation (0.1 ms pulses at 5 Hz) at *ex tempore* threshold amplitudes of between 3.0 and 5.0 V on denervated facial muscle reduces the number of innervated motor endplates in rats.<sup>47</sup> Therefore, electrical stimulation and/or electrical stimulation-associated muscle contraction is not beneficial for recovery of denervated skeletal muscle by preventing reinnervation. On the contrary, in the present study, MENS was beneficial for regrowth of atrophied muscle. The discrepancy between these results may be attributed to the strength and amount of electrical current on skeletal muscle. In addition, it is generally accepted that there is no abnormality of innervations in unloading-associated atrophied skeletal muscle. Although the effect of MENS on denervated skeletal muscle remains unclear, muscle innervation might be one of key factors for the beneficial effects of MENS on skeletal muscle.

In conclusion, the present study demonstrated that MENS facilitated regrowth of unloading-induced atrophied soleus muscle. Decrease in the reloading-induced necrosis of atrophied soleus was also facilitated by MENS. MENS might be one of extracellular stimuli to induce intracellular signals involved in protein synthesis of atrophied and/or injured skeletal muscle. Application of MENS on skeletal muscle may be an effective rehabilitation tool for atrophied and injured skeletal muscles.

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## Competing Interests

The authors have declared that no competing interest exists.

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