Regeneration of Injured Tibialis Anterior Muscle in Mice in Response to Microcurrent Electrical Neuromuscular Stimulation with or without Icing

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Abstract

Objective: Cold therapy, so-called icing, is often used immediately after muscle injuries as first aid for the suppression of inflammation and pain relief. Recent evidences show icing retards the regeneration of injured skeletal muscle. On the other hand, microcurrent electrical neuromuscular stimulation (MENS) promotes the regeneration of injured skeletal muscle. In this study, we investigated the effects of the MENS with or without icing on the regeneration of injured skeletal muscle.

Methods: Eight-week-old male mice (C57BL/6J) were divided into 4 groups: control (C), cardiotoxin (CTX) injected (X), CTX injected with MENS (XM), and CTX injected with combined treatments with icing and MENS (XIM) groups. Necrosis-regeneration cycle was induced by an intramuscular injection of CTX into tibialis anterior (TA) muscles except for C group. After CTX-injection, the hindlimbs of the mice were soaked in ice-cold water (4ºC) for 20 minutes under anesthesia (XIM). After the treatments, both right and left hindlimbs of the mice in XIM and XM groups were treated with MENS (10 µA, 0.3 Hz, 250 msec) for 60 min a day and 3 days per week for 1 or 3 weeks. One and three weeks after CTX injection, TA muscles were dissected.

Results: MENS with or without icing facilitated the recovery of muscle protein content and muscle fiber morphology including mean fiber cross-sectional areas of injured TA muscle, compared with non-treated condition. These facilitating effects of MENS with or without icing were accompanied with the increase in the relative number of Pax7-positive nuclei, namely satellite cells. Judging from fiber morphology, MENS with icing had enhanced stimulating effects on the regeneration of injured skeletal muscle, compared with MENS-treated condition.

Conclusion: Evidence suggested that MENS with or without icing facilitated the regeneration of injured TA muscle. A combination treatment of MENS with icing might be a useful therapy for sports-related skeletal muscle injuries.

Key words

muscle injury, microcurrent electrical neuromuscular stimulation, icing, satellite cell

Introduction

Skeletal muscle injuries are common sports-related injuries, and several months are required for return to normal function and activity¹², causing concern for athletes. A great deal of clinicians and researchers have focused on the promotion of repair and regeneration in injured skeletal muscle³–⁸. Non-
pharmacological treatments including heat and cold therapies have been applied to facilitate the regeneration of injured skeletal muscle. Cold therapy, so-called icing, is often used immediately after the injury as first aid.

Icing is generally considered to be useful for pain relief and anti-inflammation (swelling and bleeding). On the other hand, both positive and negative effects of icing on skeletal muscle injury have been reported. Although icing suppresses inflammation, it delays muscle regeneration. Therefore, even though icing is considered essential for first aid to reduce swelling and pain immediately after muscle injury, the physiological effects of icing on injured skeletal muscle are controversial.

Microcurrent electrical neuromuscular stimulation (MENS) has been used as one of the treatment methods for sports-related skeletal muscle injury. Results suggest that MENS offers pain relief and promotes soft tissue repair. Furthermore, it has been also reported that MENS facilitates the regeneration of tendon injuries, skin ulcers, wounds, bedsores, ligament injuries, and skeletal muscle injuries.

Considering the effects of both therapies, a combined therapy of MENS with icing may be useful for the regeneration of injured skeletal muscle. However, there is no evidence for the effects of the combined therapy. In the present study, we investigated the effects of MENS with or without icing on the regeneration of injured skeletal muscle.

**Materials and Methods**

**Animals**

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) and were approved by the Animal Experiment Committee of the Experimental Animal Research Facilities, St. Marianna University School of Medicine.

Forty-eight 7-week-old male C57BL/6J mice (Japan SLC Inc., Hamamatsu, Japan) were used. Mice were housed in an environmentally controlled room (24 ± 1 °C and 55 ± 10% relative humidity) with a 12/12 hour light-dark cycle. Animals were fed standard rat chow and water ad libitum. After a week of acclimation to the animal facility, mice were randomly divided into 4 groups: control (C), cardiotoxin (CTX) injected (X), CTX injected with MENS (XM), and CTX injected with a combined treatment of icing and MENS (XIM) groups (n=12 in each group).

**Skeletal muscle injury model**

The necrosis-regeneration cycle was induced by an intramuscular injection of CTX (100 µl, 10 µM in saline, Latoxan, France) into the proximal, middle, and distal regions of both the right and left tibialis anterior (TA) muscles except for C group, using a 27-gauge needle under anesthesia with the mice breathing isoflurane as described earlier. The same volume of physiological saline was injected into both right and left TA muscles of mice in C group. This procedure for the initiation of necrosis-regeneration was performed carefully to avoid the damage in the nerves and blood vessels, as was suggested elsewhere.

**Icing treatment**

After CTX–injection, both right and left hindlimbs of mice were completely soaked in ice-cold water (4°C) for 20 minutes under anesthesia with isoflurane in the XIM groups as described previously. During icing, the abdomen of each mouse was wrapped with an expanded polystyrene board and the tail was clipped to the back to avoid direct contact with cold water. In a pilot study, time course of the temperature of TA muscle and the esophagus in mice were monitored with a thermocouple thermometer PTW-301 (Unique Medical Co., Ltd., Tokyo, Japan). The muscle temperature fell to ~4°C immediately after icing, and was maintained for 20 min. On the other hand, the esophageal temperature became ~30°C approximately 15 minutes after the initiation of icing.

**Microcurrent electrical neuromuscular stimulation (MENS)**

After icing treatment, both right and left hindlimbs of mice in XIM and XM groups were treated with MENS (10 µA, 0.3 Hz, 250 msec) using an electrical stimulator (Trio300, Ito Co., Ltd., Tokyo, Japan) for 60 min a day and 3 days per week for 1 or 3 weeks under an anesthesia with isoflurane as described in a previous study (Figure 1). Mice of X group also received the anesthesia without MENS. Before MENS treatment, the epilation of both right and left hindlimbs of mice in all groups were performed using a commercial hair remover. Then, two electrodes were placed on the distal anterior side of the knee joint and the proximal anterior side of the ankle joint, respectively.
Figure 1. Experimental protocol. C: control group, X: cardiotoxin (CTX) -injected group, XM: CTX–injected with MENS treatment group, XIM: CTX–injected with combined treatments with icing and MENS group. 1 week: one week after CTX injection, 3 weeks: three weeks after CTX injection. ▽: CTX injection, ▲: icing, ●: sampling, ↑: MENS (dotted line).

Sampling
One and three weeks after CTX injection, mice in all groups were sacrificed under anesthesia with isoflurane. TA muscles were dissected from both hindlimbs and rapidly weighed (wet weight) and divided into three portions cross-sectionally. Then, muscle sections were frozen in isopentane cooled by liquid nitrogen and stored at -80ºC until analysis (Figure 1).

Histological and immunohistochemical analyses
Serial transverse cryosections (10-µm thick) of the frozen central portion of TA muscles were cut at -20ºC using a cryostat (CM1900, Leica, Wetzlar, Germany) and mounted on the slide glasses. The sections were air-dried and stained to evaluate the histological stage using hematoxylin and eosin (HE), and the profiles of Pax7-positive nuclei by the standard immunohistochemical technique. Since Pax7 is a specific marker for muscle satellite cells, cryosections were stained with mouse monoclonal anti-chicken Pax7 (1:5, Developmental studies Hybridoma Bank, Iowa, IA, USA). Cryosections were fixed with paraformaldehyde (4%) at 4ºC for 15 minutes. After blocking for 7 minutes using a reagent (Large Volume Ultra V Block, Thermo Scientific, Cheshire, UK), samples were incubated with the primary antibodies for Pax7 and rabbit polyclonal anti-human laminin (1:1000, Z0097, Dako Cytomation, Glostrup, Denmark) at room temperature for 3 hours. Laminin immunohistochemical staining was performed to detect basement membrane. Sections were incubated with secondary antibodies, Alexa Fluor 568-labeled goat anti-mouse IgG1 (1:500; Invitrogen, Molecular Probes, Eugene, OR, USA) and Alexa Fluor 488-labeled goat anti-rabbit IgG (1:1000; Invitrogen, Molecular Probes, Eugene, OR, USA). Then, nuclei were stained by a 5 -minute incubation in a solution of 4’,6-diamidino-2-phenylindole (DAPI, 1:10000; Sigma-Aldrich, St Louis, MO, USA) (Figure 2).

Muscle protein content
A part of the distal portion of TA muscles were re-weighted and homogenized in ice-cold RIPA (Radio-Immunoprecipitation Assay) buffer (Thermo Fisher Scientific, Waltham, MA, USA), and then completely solubilized by alkaline treatment with 2 N NaOH at 37ºC for 1 hour. Protein concentrations of homogenate samples were measured using a BCA protein assay kit (PIERCE, Pittsburgh, PA, USA) and bovine serum albumin as the standard. Protein content per milligram of TA muscle wet weight was calculated, and then absolute protein content in the whole muscle was calculated using the whole TA wet weight, according to a previous study.

Western blotting
The remaining portion of TA muscle was homogenized on ice with RIPA buffer (Thermo Fisher Scientific, MA, USA) supplemented with protease and phosphatase inhibitors (Roche Diagnostics, Manheim, Germany). Proteins were loaded onto 10% SDS-PAGE gels and run at 200 V for 30 minutes. Following SDS-PAGE, proteins were transferred onto nitrocellulose membranes using a Bio-Rad Mini Trans-Blot cell at 100 V for 60 min at 4ºC. Then, the
membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween (TBST). After blocking, membranes were incubated with the primary antibodies: rabbit polyclonal anti-mouse phosphorylated Ser\textsuperscript{473} Akt (1:2000; Cell Signaling, #9271, Beverly, MA, USA) and rabbit polyclonal anti-mouse Akt (1:2000; Cell Signaling, #9272), and then reacted with rabbit horseradish peroxidase-conjugated secondary antibodies (1:5000; Cell Signaling, #7074) for 1 hour at room temperature. After several washes, the protein bands were visualized using chemiluminescence reagent (ECL Plus Western blotting detection reagents: GE Healthcare, Tokyo, Japan) and captured with a LAS-3000 imaging system (Fuji film, Tokyo, Japan). The signal density of the protein bands was evaluated using Image J (Ver. 1.45i).

Statistical analyses

All values were expressed as means ± SEMs. Statistical significance for each measurement was analyzed by using two-way (treatment x time) ANOVA. When a significant interaction between two main factors was observed, Tukey post hoc test was carried out. The significant level of Akt expression was tested by Student t-test. All of the statistical analyses were performed using SPSS Statistics 20.0J (IBM Japan, Tokyo, Japan). The significance level was accepted at p<0.05.

Results

Muscle wet weight and protein content

Muscle wet weight relative to body weights one and three weeks after CTX injection are shown in Figure 3. At both one and three weeks, significant main effects (treatment and time) and interactions (treatment x time) were observed (p<0.05). Relative muscle wet weight in X and XIM groups were significantly lower than that in C group one week after the injection. Three weeks after CTX injection, muscle wet weight in X, XM, and XIM groups were significantly higher than that in C group (p<0.05). However, there was no significant difference in muscle wet weight among CTX–injected groups.

There were significant main effects (treatment) and interactions (treatment x time) in muscle protein content one and three weeks after CTX injection (Figure 4, p<0.05). Muscle protein content in X group significantly decreased, compared with group C (p<0.05). Three weeks after the injection, there was no significant difference in muscle protein content among the 4 groups.
**Figure 3.** Muscle wet weight relative to body weight.
Values are means ± SEMs. n=6 in each group.
a and b: significant main effects of treatment (a) and time (b) (p<0.05, analyzed by two-way ANOVA (treatment x time), c: significant interaction between two main effects (p<0.05) analyzed by two-way ANOVA (treatment x time), *: p<0.05
Abbreviations are the same as in Figure 1.

**Figure 4.** Muscle protein content relative to body weight.
Values are means ± SEMs. n=6 in each group.
Abbreviations are the same as in Figures 1 and 3.

**Morphology of muscle fibers**

**Figure 5** shows the morphological responses of CTX-injected muscles. One week after the injection, infiltrating cells were noted in X and XM groups (**Figure 5A**). In XIM group, however, many regenerating fibers with central nuclei were observed. In X and XM groups, regenerating fibers with a small diameter and central nucleus were seen even after 3 weeks, compared with XIM group. Three weeks after CTX injection, the number of fibers with a central nucleus decreased and fiber diameter increased, compared with X and XM groups (**Figure 5B**).

Mean CSAs of fibers having a central nucleus are shown in **Figure 6**. There were significant main effects (treatment and time) and interactions (treatment x time) in the mean fiber CSAs one and three weeks after the injection (p<0.05). One week after the injection, the number of fiber CSAs in X, XM, and XIM groups was significantly lower than that in group C (p<0.05). Furthermore, there were significant differences in mean fiber CSAs between X and XIM, and XM and XIM, respectively (p<0.05). Three
Figure 5. Transverse cryosections of midbelly region of mouse tibialis anterior muscle stained with hematoxylin and eosin at 1W (A) and 3W (B). Abbreviations are the same as in Figure 1.
weeks after CTX injection, the number of mean fiber CSAs in X and XM groups was also significantly lower than that in C group (p<0.05). However, there were no significant differences in mean fiber CSAs between C and XIM, and between XM and XIM.

**Number of Pax7-positive nuclei**

Figure 7 shows the changes in the number of pax7-positive nuclei relative to total myonuclei in response to CTX injection. There were significant main effects (treatment and time) and interactions (treatment × time) in the relative population of Pax7-positive nuclei (p<0.05). The relative number of Pax7-positive nuclei in X, XM, and XIM groups was significantly higher than that in C group one and three weeks after CTX injection (p<0.05). One week after the injection, the relative number of Pax7-positive nuclei in XM and XIM groups was also significantly higher than that in X group. There were significant differences in the relative number of Pax7-positive nuclei between X and XIM, and between XM and XIM groups at three weeks (p<0.05).
Akt expression

In this study, the activation level of Akt was evaluated using the phosphorylated level of Akt relative to the total expression form (p-Akt/t-Akt), since phosphorylated form of Akt is enzymatically active⁴³⁴⁴. Figure 8 shows the relative expression levels of p-Akt to t-Akt one week after CTX injection in XM and XIM groups. The relative expression level of p-Akt in XIM was higher than that in XM group (p=0.059).

Discussion

In the present study, we investigated the effects of MENS treatments with or without cold therapy on the regeneration of injured TA muscle in mice. Results showed that MENS with or without icing facilitated the recovery of muscle protein content and muscle fiber morphology including mean fiber CSAs of injured TA muscle, compared with non-MENS-treatment. The population of Pax7-positive nuclei, namely satellite cells, was increased by MENS with or without icing. Judging from fiber morphology, MENS with icing had enhanced stimulating effects on the regeneration of injured skeletal muscle, compared with MENS without icing. The expression level of p-Akt in MENS with icing was also higher than that in MENS treatment without icing.

Effects of MENS

MENS without icing facilitated the recovery of muscle wet weight, muscle protein content, and mean fiber CSAs in injured TA muscle, compared with non-treated X group. These results are consistent with the previous study²⁰. Although the molecular mechanism(s) of MENS-associated facilitation of injured skeletal muscle remain unclear, the involvement of the active transport of amino acids through increased generation of adenosine triphosphate (ATP) or elevated protein synthesis have been suggested⁴⁵⁴⁶. It has been reported that electric current increases ATP synthesis up to 500% at 500 µA in skin tissue, whereas ATP synthesis gradually decreased beyond 1,000 µA⁴⁷. Synthesis of ATP might be a cause of the facilitations of injured skeletal muscle.

MENS also increased the relative number of Pax7-positive nuclei one week after CTX injection, compared with C group. A MENS-associated increase in Pax7-positive nuclei was also observed in the previous study²⁰. The population of Pax7-positive nuclei increased in muscle following CTX-injection-associated injury³¹. In the present study, a CTX injection-associated increase in the number of Pax7-positive nuclei was also observed. One week after the injection, MENS increased the number of Pax7-positive nuclei, compared with non-treated group. MENS-associated facilitation of the regeneration of injured skeletal muscle might be attributed to the higher level of Pax7-positive nuclei. However, the molecular mechanism(s) of MENS-associated increase in Pax7-positive nuclei are unknown. Additional investigations should be needed to elucidate this point.

Combined effects of MENS with icing

In this study, the combined effects of MENS with icing were investigated. MENS with icing facilitated the regeneration of injured TA muscle, compared with non-treated X group. These facilitating effects of MENS with icing were almost identical to those of MENS alone. This is the first study to investigate the combined effects of MENS and icing on the regeneration of injured skeletal muscle. Recent evidences show that icing suppressed the regeneration of injured skeletal muscle in rats⁷ and mice⁸. Although the effects of icing alone on the regeneration
of injured TA muscle was not investigated in the present study, icing may depress the regeneration of injured TA muscle. If so, MENS following icing might diminish such negative effects on the skeletal muscle regeneration.

Histological analyses show MENS with icing stimulated the regeneration of injured TA muscle, compared with MENS alone (Figure 5). One week after CTX injection, the activation level of Akt in MENS with icing was higher than that in MENS alone. Overexpression of activated Akt promotes the regeneration of CTX injection-associated injured muscle fibers. Furthermore, three weeks after the injection, a higher level of the population of Pax7-positive nuclei in MENS with icing was observed. Although these factors may be a part of the facilitation of injured TA muscle, we have no clear explanation for the reason why the combined treatment of MENS with icing has such effects.

**Perspective**

Icing on sports-related injuries is important for both the suppression of inflammation and pain relief. Furthermore, icing-associated vasoconstriction decreases edema formation and cellular damage with hypoxia of injured tissue. On the other hand, recent evidence shows icing retards the regeneration of injured skeletal muscles. Therefore, the combination treatment of MENS with icing might be a useful therapy for sports-related skeletal muscle injuries with both the suppression of inflammation and pain relief.

**Conclusion**

The present study investigated the effects of MENS with or without icing on the regeneration of injured TA muscle in mice. Evidence suggested that MENS with or without icing facilitated the regeneration of injured TA muscle. A combination treatment of MENS with icing might be a useful therapy for sports-related skeletal muscle injuries with both the suppression of inflammation and pain relief.

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