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Research article

Microcurrent Electrical Neuromuscular Stimulation Facilitates Regeneration of Injured Skeletal Muscle in Mice

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Abstract

Conservative therapies, mainly resting care for the damaged muscle, are generally used as a treatment for skeletal muscle injuries (such as muscle fragmentation). Several past studies reported that microcurrent electrical neuromuscular stimulation (MENS) facilitates a repair of injured soft tissues and shortens the recovery period. However, the effects of MENS on the regeneration in injured skeletal muscle are still unclear. The purpose of this study was to investigate the effect of MENS on the regenerative process of injured skeletal muscle and to elucidate whether satellite cells in injured skeletal muscle are activated by MENS by using animal models. Male C57BL/6J mice, aged 7 weeks old, were used (n = 30). Mice were randomly divided into two groups: (1) cardiotoxin (CTX)-injected (CX, n = 15) and (2) CTX-injected with MENS treatment (MX, n=15) groups. CTX was injected into tibialis anterior muscle (TA) of mice in CX and MX groups to initiate the necrosis-regeneration cycle of the muscle. TA was dissected 1, 2, and 3 weeks after the injection. Muscle weight, muscle protein content, the mean cross-sectional areas of muscle fibers, the relative percentage of fibers having central nuclei, and the number of muscle satellite cells were evaluated. MENS facilitated the recovery of the muscle dry weight and protein content relative to body weight, and the mean cross-sectional areas of muscle fibers in CTX-induced injured TA muscle. The number of Pax7-positive muscle satellite cells was increased by MENS during the regenerating period. Decrease in the percentages of fibers with central nuclei after CTX-injection was facilitated by MENS. MENS may facilitate the regeneration of injured skeletal muscles by activating the regenerative potential of skeletal muscles.

Key words: Muscle injury, regenerative potential, muscle satellite cell, central nuclei, physiotherapy, sports injury.

Introduction

Skeletal muscle injury is one of the most common injuries in sports-related traumas.

The standard therapeutic methods for treating skeletal muscle injuries such as muscle fragmentation generally use conservative therapy that mainly involves resting of the damaged site. However, recovery can take over several months depending on the extent of skeletal muscle damage (Garret, 1990; Huard et al., 2002; Lehto and Jarvinen, 1991; Peterson and Renström, 2001). This is a serious problem for athletes eager for an early return. Even though significant clinical efforts are being made to improve the current treatment of skeletal muscle injuries, it is not easy to establish the treatment of accelerated recovery.

The regenerative process of injured skeletal muscle is highly coordinated and complex phenomena. Skeletal muscle-specific stem cells, so-called muscle satellite cells, are well known to be responsible for repair and regeneration of adult skeletal muscle tissues (Best and Hunter, 2000; Grounds, 1999; Hill et al., 2003; Saito and Nonaka, 1994; Seale and Rudnicki, 2000). Injured skeletal muscles in genetically developed mice lacking muscle satellite cells exhibited a severe depression of regenerative potential (Seale et al., 2000a). However, the regulatory mechanisms for the regenerative potential of injured skeletal muscle are still unclear. It has been reported that the proliferative stimuli for satellite cells is stimulated by extracellular stimuli, such as mechanical (Morioka et al., 2008) and heat (Kojima et al., 2007) stresses, resulting that such stresses facilitated the regeneration of injured rat tibialis anterior and mouse soleus muscle, respectively. On the other hand, unloading reportedly impairs the regenerative potential of atrophied soleus muscle (Matsuba et al., 2009). These observations suggest that the proliferative potential of injured skeletal muscle might be sensitive to extracellular stimuli.

Microcurrent electrical neuromuscular stimulation (MENS) was developed as a physical therapy modality delivering current in the microampere range. It has been reported that MENS has several physiological effects such as pain relief (Larner and Kirsch, 1981) and facilitation of tissue repair including tendon injuries (Nessler and Mass, 1987; Owoeye et al., 1987), skin ulcers (Gault and Gatens, 1976; Wolcott et al., 1969), wounds (Byl et al., 1994; Huckfeldt et al., 2007), bedsores (Carley and Wainpel, 1985) and ligament injuries (Miyazaki et al., 2007). Recently, we have confirmed that the regrowth of unloading-associated atrophied mouse soleus muscle is stimulated by MENS (Ohno et al., 2013). Previous studies (Curtis et al., 2010; Lambert et al., 2002) have suggested that MENS facilitates a repair of injured skeletal muscle and shortens the recovery period. However, it is still unclear whether MENS has stimulating effects on activation of regenerative potential in injured skeletal muscle.

The purpose of this study was to investigate the ef-

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fect of MENS on the regeneration process of injured skeletal muscle and to investigate whether satellite cells in injured skeletal muscle are activated by MENS. Evidences from this study suggest that MENS shortens the recovery period through the activation of satellite cells in injured skeletal muscle.

Methods

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) and were approved by the Animal Use Committee of Toyohashi SOZO University. All procedures adhered to the American College of Sports Medicine animal standards.

Male C57BL/6J mice, aged 7 weeks old, were used (n = 30). Mice were randomly divided into two groups: (1) cardiotoxin (CTX)-injected (CX, n = 15) and (2) CTX-injected with MENS treatment (MX, n = 15) groups. Five mice of both groups were housed in a home cage (20 x 31 cm and 13.5 cm height) in a clean animal 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.

Initiation of necrosis-regeneration cycle

In mice of both groups, 0.1 ml CTX (10 µM in physiological saline (PS); Sigma, St.Louis, MO, USA) of Naja naja atra venom was injected into the proximal, middle and distal part of the left tibialis anterior (TA) muscle, using a 27-gauge needle under anesthesia with sodium pentobarbital (50 mg·kg^{-1} body weight, i.p.). Injection of CTX was performed carefully to avoid the damage in the nerves, blood vessels (Conteaux et al., 1988; Fletcher and Jiang, 1993). This treatment causes the initiation of necrosis-regeneration cycle (Conteaux et al., 1988; Fletcher and Jiang, 1993), and used as a muscle injury model in previous studies (Kojima et al., 2007, Morioka et al., 2008).

MENS treatment

Forty eight hours after the CTX-injection, the left hindlimb of mice in MX group were treated with MENS (10 µA, 0.3 Hz, 250 msec) by using an electical stimulator (Trio300, Ito Co., Ltd., Tokyo) for 60 min a day and 3 days a week for 3 weeks under anesthesia with i.p. injection of sodium pentobarbital (50 mg·kg^{-1}). Mice in CX group were also anesthetized for 60 min without MENS treatment. Before MENS treatment, the epilation of mouse hindlimb was performed using a commercial hair remover for human. Then, two electrodes were placed on the distal anterior side of the knee joint and the proximal anterior side of ankle joint, respectively. In the present study, no muscle contraction in hindlimb of mice was observed during MENS. The condition for MENS was set by accounting the body size of the experimental animal and the duration of MENS treatment compared with humans which are clinically treated with MENS (Morinaga, 2007).

Sampling

TA muscles of left hindlimb in both groups were dissected 1, 2 and 3 weeks after the CTX-injection under anethsesia. TA muscles was trimmed of excess fat and connective tissues, weighted, frozen in liquid nitrogen, and stored at -80°C until analyses.

Histochemical and immunohistochemical analysis

TA muscles were cross-sectionally cut into halves at the middle of the long axis. Serial transverse cryosections (7 µm thick) of the distal region of frozen TA muscles cut at -20 ^{o}C and mounted glass slides. The sections were air-dried and stained to evaluate the pathological stages by using hematoxylin and eosin (H&E) and the profiles of Pax7-positive nuclei by using the standard immunohistochemical technique (Kojima et al., 2007), respectively. General pathological observations including centrally located myofiber nuclei were based on H&E staining. The cross-sectional area (CSA) of muscle fibers was also analyzed.

Briefly, monoclonal anti-Pax7 antibody (Developmental Studies Hybridoma Bank, Iowa, IA, USA) was used for the detection of muscle satellite cells (Asakura et al., 2002; Hawke and Garry, 2001; Morgan and Partridge, 2003; Seale et al., 2000b; 2001). Cross sections were fixed with paraformaldehyde (4%) for 15 min, and then were post-fixed in ice-cold methanol for 15 min after 3-time washing with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (TPBS). After blocking by using a reagent (1% Roche Blocking Regent; Roche Diagonost, Penzberg, Germany), samples were incubated with the primary antibodies for Pax7 and rabbit polyclonal anti-laminin (Z0097; diluted 1: 500; Dako Cytomation, Glostrup, Denmark). Thereafter, sections were also incubated with the second primary antibodies for Cy3-conjugated anti-mouse IgG1 (diluted 1: 500; Jackson Immuno Research, West Grove, PA, USA) and for fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (diluted 1: 500; Sigma, USA). Nuclei were then stained for 15-min in a solution of 2,4-diamidino-2-phenylindole dihydrochloride n-hydrate (Dapi, 0.5 mg·ml^{-1}; Sigma).

Imaging of muscular sections and analysis

The images of muscle sections were incorporated into a personal computer (DP Manager version 2.2.1.195, Olympus Japan, Tokyo) by using a microscope (IX 81 Olympus Japan). The CSA of 200~300 fibers from each muscle was analyzed by using Image J (Ver. 1.45i, Wayne Rasband, National Institutes of Health, USA). The percentage of fibers having central nuclei relative to total muscle fibers in the whole transverse section was also calculated. Both Pax7- and Dapi-positive nuclei, located within laminin-positive basal membrane, were counted in the whole transverse section and were expressed as the total number of both Pax7-positive nuclei and myonuclei per cross section of TA muscle. Furthermore, the percentage of Pax7-positive nuclei relative to the total number of Dapi-positive nuclei was calculated (Kojima et al., 2007).

Muscle dry weight and protein content

Frozen proximal pieces of TA muscle were placed in a

freeze-dryer (-45 °C) under vacuum for ~48-72hr as was reported elsewhere (Naito et al., 2000). Dry muscle tissues were then weighted. Muscle protein content was determined in proximal pieces. Briefly, the muscles were homogenized in 10 volumes of isolation buffer (10 mM Tris-HCL, 10 mM NaCl, and 0.1 mM EDTA, pH7.6), and completely solubilized by alkali treatment with 1 volume of 2 N NaOH at 37 °C for 30 min. Protein concentration in the homogenates was determined by using the protein assay kit (Bio-Rad, Hercules, CA, USA) and bovine serum albmin (Sigma) as the standard. Total protein content in whole muscle relative to body weight was also calculated (Kojima et al., 2007).

Statistical analysis

All values were expressed as mean±SEM. Statistical significance was analyzed by using two-way (treatment x time) ANOVA. When a significant main effect was observed, Tukey post hoc test was performed in each group. When a significant interaction between two effects (treatment and time) was observed, Tukey post hoc test was performed in each experiment. Statistical significance was established at $p < 0.05$.

Results

Muscle dry weight and protein content

Body and the absolute TA muscle (wet and dry) weights are shown in Figures 1A (body weight), 1B (absolute muscle wet weight), and 1C (absolute muscle dry weight), respectively. No significant main effect was observed by two-way ANOVA (treatment x time) for the changes in the body weight. Two-way ANOVA (treatment x time) for the changes in the absolute muscle wet and dry weight revealed significant main effects of time ($p < 0.05$, $p < 0.05$). Mean values of muscle dry weight and protein content relative to body weight are shown in Figures 1D and 1E, respectively. In both groups, a trend to increase in the relative muscle dry weight and protein content was observed during the experimental period. Two-way ANOVA (treatment x time) for the changes in the relative muscle dry weight revealed significant main effects of treatment ($p < 0.05$) and time ($p < 0.05$). Regarding the protein content, two-way ANOVA revealed a significant effect of time ($p < 0.05$), but no effect of treatment ($p = 0.78$). A significant interaction of treatment and time was observed ($p < 0.05$). According to the post-hoc test, mean levels of muscle protein content were lowest in 1-week, intermediate in 2-week, and highest in 3-week after the CTX-injection.

Histological analysis

The H&E stained images showed that TA muscle had many regenerating fibers at 1 week after the CTX-injection (Figure 2). Small fibers with central nuclei were observed 1 and 2 weeks after the CTX-injection in both CX and MX groups.

The percentages of fibers with central nuclei after the CTX-injection are shown in Figure 3. There were significant main effects of time ($p < 0.05$) and treatment ($p < 0.05$) in the percentage of fibers with central nuclei.

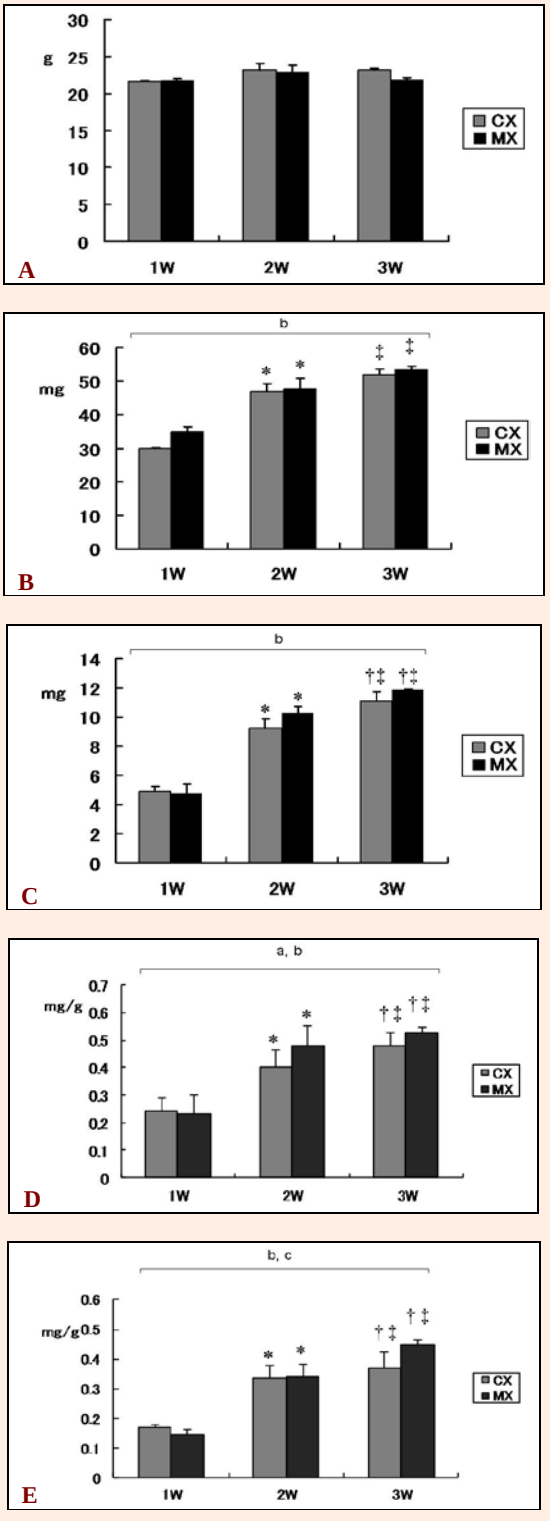

Figure 1. Body weight (A), absolute muscle wet weight (B), absolute muscle dry weight (C), muscle dry weight relative to body weight (D), and muscle protein content relative to body weight (E). CX: cardiotoxin (CTX)-injected group, MX: CTX-injected with MENS treatment group. Values are means ± SEM. Units are mg/g. n=5 in each group. When the significant main effect (treatment or time) analyzed by using two-way ANOVA (treatment x time) was observed, the results were shown using "a" (treatment) or "b" (time). When a significant interaction between two effects (treatment and time) were observed, the results were shown using "c". Significant differences of the data between 1 and 2 weeks, 2 and 3 weeks and 1 and 3 weeks were shown using *, †, and ‡, ($p < 0.05$) , respectively.

There was a significant difference in the percentages of fibers with central nuclei between 1 and 3 weeks ($p < 0.05$). The mean percentage of these fibers in CX group at 1 week after the injection was ~95% (Figure 3). Although the mean percentage of fibers with central nuclei in CX group showed a trend to decrease during the experimental period, the value at 3 weeks after the CTX-injection in CX group was ~88%. On the other hand, in MX group, the mean value of fibers with central nuclei was trended to be lower than that in CX group 2 weeks after the CTX-injection ($p < 0.05$).

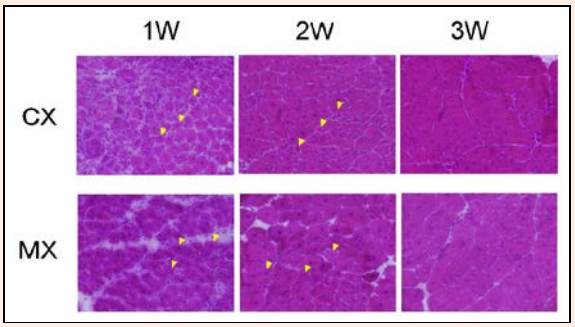

Figure 2. Typical images of injured skeletal muscle during the regeneration. Cross-sections were stained with H&E. Arrow heads show small fibers with central nuclei. CX: cardiotoxin (CTX)-injected group, MX: CTX-injected with MENS treatment group.

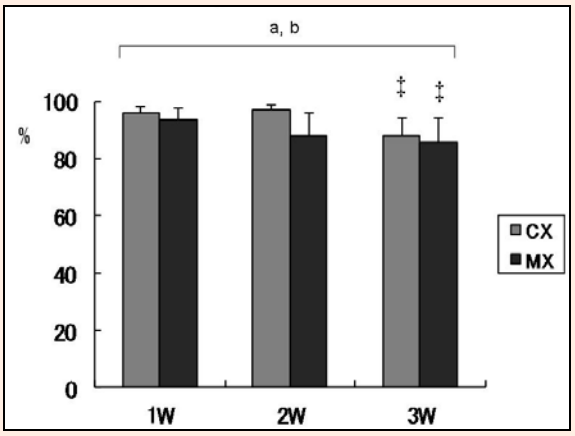

Figure 3. Relative percentages of fibers with central nuclei. CX: cardiotoxin (CTX)-injected group, MX: CTX-injected with MENS treatment group. Values are means ± SEM. n=5 in each group. When the significant main effect (treatment or time) analyzed by using two-way ANOVA (treatment x time) was observed, the results were shown using "a" (treatment) or "b" (time). Significant differences of the data between 1 and 3 weeks were shown using ‡, ($p < 0.05$).

Figure 4 shows the mean cross-sectional area of muscle fibers in TA muscle. The mean cross-sectional area in both CX and MX groups was gradually increased during the experimental period. There were significant main effects (treatment and time) and a significant interaction between treatment and time in the cross-sectional area of muscle fibers ($p < 0.05$). There were significant differences between 1 and 2 weeks, 2 and 3 weeks, and 1 and 3 weeks after the CTX injection ($p < 0.05$) in both CX and MX group. In MX group, the mean cross-sectional area of muscle fibers was higher than those in CX group.

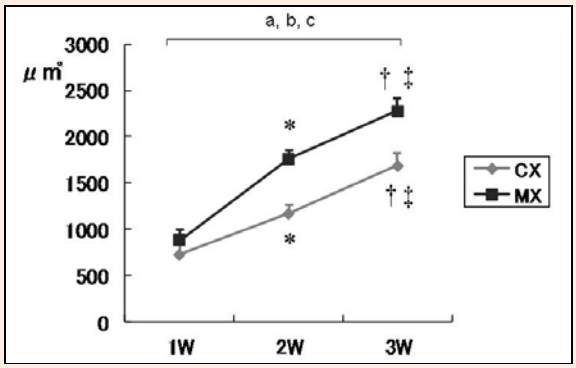

Figure 4. Mean cross-sectional area of muscle fibers. CX: cardiotoxin (CTX)-injected group, MX: CTX-injected with MENS treatment group. Values are means ± SEM. Units are μm^2. n=5 in each group. When the significant main effect (treatment or time) analyzed by using two-way ANOVA (treatment x time) was observed, the results were shown using "a" (treatment) or "b" (time). When a significant interaction between two effects (treatment and time) were observed, the results were shown using "c". Significant differences of the data between 1 and 2 weeks, 2 and 3 weeks and 1 and 3 weeks were shown using *, †, and ‡, ($p < 0.05$) , respectively.

Myonuclei and pax7-positive nuclei

The number of Pax7-positive nuclei and myonuclei on the cross-section of TA muscle were shown in Figures 5A and 5B, respectively. Figure 5C shows the number of Pax7-positive nuclei relative to total myonuclei. There were significant main effects (treatment and time) and a significant interaction between treatment and time in both the absolute and the relative number of Pax7-positive nuclei to myonuclei ($p < 0.05$) (Figures 5A and 5C). The total numbers of myonuclei in MX group tended to be higher those in CX group during the experimental period (Figure 5B). The total and relative numbers of Pax7-positive nuclei in both CX and MX groups showed a trend to decrease during the experimental period (Figures 5A and 5C). In MX group, both number of Pax7-positive nuclei and relative to myonuclei were higher those in CX group.

Discussion

The present study indicated MENS facilitated the recovery of the muscle dry weight and protein content relative to body weight, and the mean cross-sectional areas of muscle fibers of CTX-induced injured TA muscle. MENS-associated increase in the number of Pax7-positive muscle satellite cells was also observed during the regenerating period. Decrease in the percentages of fibers with central nuclei after CTX-injection was facilitated by MENS. This is the first study showing that the effects of MENS on the regeneration of injured skeletal muscle in an animal model.

In this study, the relative muscle dry weight and the relative protein content during the regenerating phase of injured TA muscle were increased by MENS treatment. These results suggest MENS treatment stimulated protein synthesis of injured skeletal muscle during the regenerating period. In the present study, gradual increase in the

mean cross-sectional area of both CTX-injected groups was observed during the experimental period (Figure 4). The mean cross-sectional area of regenerating skeletal muscle fibers was gradually increased after muscle injury (Morioka et al., 2008; Matsuba et al., 2009; Nishizawa et al., 2013). In addition, the present study showed that the recovery of the mean cross-sectional area of injured fibers was facilitated by MENS. It has been reported that the application of MENS on tissues stimulates ATP synthesis (Cheng et al., 1982) via the facilitation of electron transport chain in the mitochondria (Miyazaki et al., 2007). However, we have no clear explanation regarding MENS-associated stimulation of recovery of muscle fiber size in injured skeletal muscle, at present.

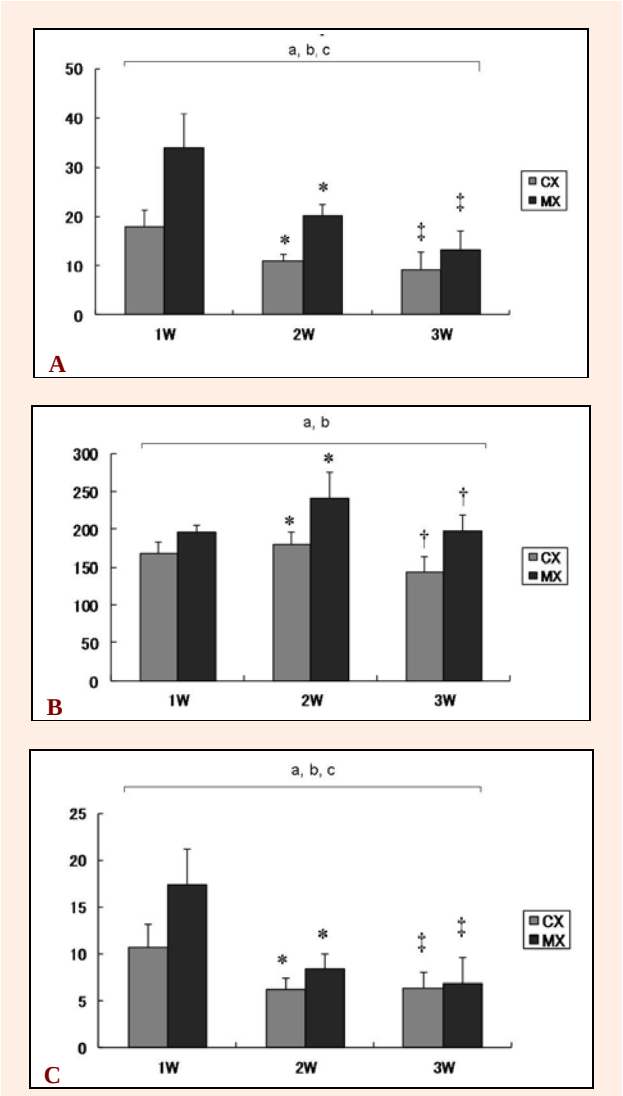

Figure 5. The number of Pax7-positive nuclei (A), the number of myonuclei (B), and the number of Pax7-positive nuclei relative to myonuclei (C). CX: cardiotoxin (CTX)-injected group, MX: CTX-injected with MENS treatment group. Values are means ± SEM. n=5 in each group. When the significant main effect (treatment or time) analyzed by using two-way ANOVA (treatment x time) was observed, the results were shown using "a" (treatment) or "b" (time). When a significant interaction between two effects (treatment and time) were observed, the results were shown using "c". Significant differences of the data between 1 and 2 weeks, 2 and 3 weeks and 1 and 3 weeks were shown using *, †, and ‡, ($p < 0.05$) , respectively.

The central nucleus is an indicator of myofiber regeneration (Chargé and Rudnicki, 2004; Morioka et al., 2008; Matsuba et al., 2009; Tidball and Wehling-Henricks, 2007). The number of fibers with central nuclei is gradually decreased thereafter (Reed and Bloch, 2005; Turgeman et al., 2008). Therefore, the number of the fibers with central nuclei was measured as an indicator for the recovery from muscle injury in the present study. In this study, the mean percentage of the fibers with central nuclei was ~95% and ~97% at 1 and 2 weeks after CTX-injection, and then gradually decreased to ~88% after 3 weeks of recovery. On the other hand, the relative number of these fibers was ~93%, ~88%, and ~85% at 1, 2, and 3 weeks after the recovery with MENS. These observations suggest that the regeneration of injured skeletal muscle was facilitated by MENS.

In the present study, Pax7-positive satellite cells in the regenerating skeletal muscle are increased by MENS treatment. This is the first study showing that the effects of MENS treatment on the number of satellite cells. Though the proliferative stimuli for satellite cells, such as mechanical (Goto et al., 2003; Morioka et al., 2008) and heat (Goto et al., 2003; Kojima et al., 2007) stresses, are well known, the molecular mechanisms for MENS-associated increase in Pax7-positive satellite cells of injured skeletal muscle is unclear. It has been reported that the electrical stimulation (2-20 Hz, 0.5-20 mA) provides an effective stimulus to rescue the loss of myonuclei and satellite cells in disuse muscle atrophy in mice (Guo et al., 2012). Although MENS, which was used in the present study (0.3Hz, 10 μA) is different from the electrical stimulation (2-20Hz), MENS also has a similar effect on muscle satellite cells. Additional studies are needed to elucidate this issue.

Conclusion

The present study strongly suggested that MENS has an effect to facilitate the regeneration of injured skeletal muscles via stimulations the proliferative potential of muscle satellite cells. MENS may be a useful therapeutic method for injured skeletal muscle. The further studies to determine the conditions of MENS should be needed for clinical applications.

Acknowledgments

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Key points

- Microcurrent electrical neuromuscular stimulation (MENS) facilitated the recovery of the relative muscle dry weight, the relative muscle protein content, and the mean cross-sectional areas of muscle fibers of injured TA muscle in mice.
- The number of satellite cells was increased by MENS during the regenerating phase of injured skeletal muscle.
- Decrease in the percentages of fibers with central nuclei was facilitated by MENS.
- MENS may facilitate the regeneration of injured skeletal muscles.

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