Effect of Combination Microcurrent Electrical Neuromuscular Stimulation and Hyperbaric Oxygen Therapy on the Regeneration of Injured Skeletal Muscle in Mice

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Abstract

We investigated, in an animal model of skeletal muscle injury, whether microcurrent electrical neuromuscular stimulation (MENS) and hyperbaric oxygen (HBO) therapy used in combination (MENS and HBO) enhances the restorative benefits conferred by these treatments alone. Skeletal muscle injury was simulated in 40 7-week-old male C57BL/6J mice by injection of cardiotoxin (CTX) into the left tibialis anterior (TA) muscle of each mouse, which induced the necrosis-regeneration cycle. The mice were divided into 4 groups of 10 each and subjected to one of the following no additional treatment (X), HBO (XH), MENS (XM), MENS and HBO (XMH) groups. MENS was delivered at 10 µA intensity, 0.3 Hz frequency, and 250 msec pulse width for 60 minutes per day, 3 days per week for 2 weeks, and HBO was performed with 100% oxygen at 2 atmospheres absolute (ATA) for 60 minutes per day, 3 days per week for 2 weeks. One and 2 weeks after CTX injection, the TA muscle was dissected. Two weeks after CTX injection, both the mean fiber cross-sectional area (CSA) and percentage increase in the fiber CSA were significantly greater in the XMH group than in the X group (P <0.05). The fiber CSA increased without treatment and also under sole treatment with either MENS or HBO, but the values did not differ significantly. Our data indicate that combination MENS and HBO therapy enhances regeneration of injured skeletal muscle and that such combination therapy may be clinically beneficial.

Key words

Muscle injury, microcurrent electrical neuromuscular stimulation, hyperbaric oxygen therapy, regeneration

Introduction

Sports-related skeletal muscle injury resulting from trauma leads to a decline in athletes’ performance and even eventual withdrawal from competition. The most desirable treatment will shorten the recovery period and allow the athlete to return to competition fairly rapidly.

The standard approach to skeletal muscle injury is conservative treatment involving mainly rest and/or cold therapy, but a return to competition following this type of treatment is frustratingly slow. A relatively new means of physical therapy, microcurrent electrical neuromuscular stimulation (MENS), has made it possible for athletes with skeletal muscle injury to return to competition more quickly. The MENS current is in the microampere (µA) range. It has no known side effects, and it can be delivered with a compact device1). There have been reports that MENS facilitates the repair of soft tissue in the context of skeletal muscle injury2–6), ligament injury7), tendon injury8,9), wound10), skin ulcer11), and bed-
sore\textsuperscript{12}).

Fujiya et al.\textsuperscript{2)} showed, in a study of injured skeletal muscle in mice, that MENS facilitated recovery of the muscle mass and fiber cross-sectional area (CSA). Further, there has been some thought that MENS combined with icing might be a useful therapy for sports-related skeletal muscle injuries\textsuperscript{31}.

Hyperbaric oxygen (HBO) therapy is known to facilitate repair of damaged tissue\textsuperscript{13–28}. With HBO, pure oxygen is administered in a high pressure environment of 2.0 or more atmospheres absolute (ATA) for 60 minutes. It is thought that HBO facilitates the regeneration of injured skeletal muscle by raising the lowered O\textsubscript{2} level\textsuperscript{29\textsuperscript{30}}. In injured skeletal muscle, circulatory disturbance is accompanied by an increase in extracellular fluid and a low-oxygen environment\textsuperscript{31\textsuperscript{32}}. Partial pressure of oxygen (PaO\textsubscript{2}) is ~40–60 mmHg in uninjured skeletal muscle and subcutaneous tissue and ~15 mmHg in damaged tissue\textsuperscript{27}. High pressure at 2.0 ATA causes an increase in the dissolved O\textsubscript{2} in the plasma. Therefore, HBO increases the O\textsubscript{2} level in injured skeletal muscle\textsuperscript{16\textsuperscript{20}}, which in turn improves permeability of the peripheral blood vessels and decreases the injury-induced swelling\textsuperscript{29\textsuperscript{30}}.

In general, HBO is considered to be applicable to emergency conditions (carbon monoxide poisoning, severe infection, compartment syndrome, for example) and to non-emergency conditions (refractory ulcer, skin graft, chronic refractory osteomyelitis, for example)\textsuperscript{33\textsuperscript{34}}. There have been numerous reports indicating that HBO facilitates the regeneration of soft tissue\textsuperscript{13–28}, and this includes the regeneration required in cases of skeletal muscle injury\textsuperscript{19–24}. The beneficial effects of HBO have been reported in relation to various specific sports injuries\textsuperscript{21\textsuperscript{22}\textsuperscript{25–28}}.

Even though both MENS and HBO promote the regeneration of injured skeletal muscle, MENS plus HBO has not been tested in the context of skeletal muscle injury. The purpose of this study was to investigate, in an animal model, the effect of MENS plus HBO on injured skeletal muscle. The skeletal muscle regeneration process consists of at least 2 phases: the initial necrosis and inflammation phase and the subsequent muscle fiber formation phase, and we focused on these 2 phases.

**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, USA), and all were approved by the Animal Experiment Committee of the Experimental Animal Research Facilities, St. Marianna University School of Medicine (Kawasaki, Japan).

Forty 7-week-old male C57BL/6J mice (Japan SLC Inc., Hamamatsu, Japan) were used in the study. The mice were housed in an environmentally controlled room (24±1°C and 55±10% humidity) under a 12/12-hour light/dark cycle. They were fed standard rat chow and water ad libitum.

**Skeletal muscle injury model and division of animals into groups**

Skeletal muscle injury was simulated in all mice by intramuscular injection of 0.1ml cardiotoxin (CTX) (10 µM in saline; Latoxan, Portes-lès-Valence, France) into the proximal, middle, and distal regions of the left tibialis anterior (TA) muscle\textsuperscript{23\textsuperscript{31}}. Anesthesia was induced with isoflurane gas, and the CTX was then injected with a 27-gauge needle. The CTX injection, which initiated the necrosis-regeneration cycle, was performed carefully to avoid damage to the nerves and blood vessels\textsuperscript{35\textsuperscript{36}}. To clarify the effects of MENS plus HBO on muscle regeneration, the CTX-injected mice were randomly divided between 4 post-CTX injection strategies: no treatment (X group, n=10), HBO (XH group, n=10), MENS (XM group, n=10) and MENS plus HBO (XMH group, n=10).

**MENS**

MENS was applied to the left hindlimb of each mouse in the XM group and XMH group. An electrical stimulator (Trio300, Ito Co., Ltd., Tokyo, Japan) was used to deliver current (10 µA intensity, 0.3 Hz frequency, 250 msec pulse width) for 60 minutes per day, 3 days per week for 1 week (5 mice per group) or 2 weeks (5 mice per group). This procedure was done under isoflurane anesthesia. Before the MENS, electrodes were attached to each animal’s left hindlimb, commercial hair remover cream was applied at the attachment sites, which were the distal surface of the knee joint and proximal surface of the ankle joint.

**HBO**

HBO was applied to each mouse in the XH group and XMH group. The mice were placed in a 15.0-L cylindrical pressure chamber (P-5100 S, Barotec Hanyuda Co., Ltd., Tokyo, Japan) and exposed to
100% oxygen at 2.0 ATA for 60 minutes per day, 3 days per week for 1 week (5 mice per group) or 2 weeks (5 mice per group).

**Tissue sampling**

Because we were interested in the initial stage of regeneration of skeletal muscle after the injury, mice in all 4 groups were sacrificed 1 week (5 mice in each group) and 2 weeks (5 mice in each group) after the CTX injection, and we dissected the TA muscle from the hindlimb. The TA muscle was then trimmed of excess fat and connective tissues, and rapidly weighed (wet weight). The dissected muscle was frozen in liquid nitrogen and stored at −80°C until analysis. The experimental protocol is diagrammed in Figure 1.

**Histological analyses**

Serial transverse sections (10 μm thick) of the central portion of the frozen TA muscle were cut at −20°C with a cryostat (CM1900, Leica, Wetzlar, Germany), and these cryosections were mounted on glass slides. The sections were then air-dried and stained with hematoxylin and eosin (HE) for evaluation of the histologic stage, which was done under a KEYENCE BZ-9000 microscope (KEYENCE, Osaka, Japan). Several images of each section were captured. The CSA of approximately 300 fibers with central nuclei was measured with the use of Image J (Ver. 1.45i, Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). The increase in fiber CSA from 1 to 2 weeks after CTX injection was determined, and then the percentage increase was calculated as (fiber CSA at 2 weeks after CTX injection − amount of increase in fiber CSA) / (fiber CSA at 1 week after CTX injection) x 100 (%).

**Statistical analyses**

Values are expressed as mean ± SEM. Significant difference was analyzed using two-way (treatment × time) analysis of variance (ANOVA) were performed followed by Tukey HSD post hoc test. Tukey HSD was employed to evaluate the effects of treatment within each level of “time” factor. All statistical analyses were performed with SPSS Statistics 21.0J (IBM Japan, Tokyo, Japan), and P <0.05 was considered significant.

**Results**

**Absolute muscle wet weight**

Absolute TA muscle wet weight at 1 and 2 weeks after CTX injection is shown per group in Figure 2. Neither treatment nor time was shown to have a significant effect on absolute TA muscle wet weight in any group of mice, and no significant interaction was found.

**Muscle wet weight relative to body weight**

TA muscle wet weight relative to body weight at 1 and 2 weeks after CTX injection is shown per group in Figure 3. Neither treatment nor time was shown to have a significant effect on TA muscle wet weight relative to body weight in any group of mice, and no significant interaction was found with respect to time. The wet weight of TA muscle relative to body weight at 1 and 2 weeks after CTX injection is shown per group in Figure 4. Neither treatment nor time was shown to have a significant effect on TA muscle wet weight relative to body weight in any group of mice, and no significant interaction was found with respect to time.
to TA muscle wet weight relative to body weight.

**Histological findings**

Examples of HE-stained cryosections of TA muscle obtained at 1 week and 2 weeks after CTX injection are shown in Figure 4A and B, respectively. At 1 week, infiltrating cells were noted in sections from the X group, and many regenerating fibers with central nuclei were observed in sections from the XH, XM and XMH groups (Figure 4A). At 2 weeks, the populations of infiltrating cells and muscle fibers with central nuclei were decreased in the XMH group (Figure 4B). In addition, diameter of the fibers appeared larger in the XMH group than in the X, XH and XM groups (Figure 4B).

**Fiber CSA**

Mean fiber CSA (i.e., CSA of fibers with central nuclei) of the TA muscle is shown per group in Figure 5. At 1 week after CTX injection, mean fiber CSA did not differ significantly between the 4 groups. However, at 2 weeks, mean fiber CSA was significantly greater in the XMH group than in the X group ($P<0.05$). Furthermore, mean fiber CSA in the XH group and XM group increased by 120% and 123%, respectively, whereas that in the X group increased. There was no significant difference in the mean fiber CSA between the XH, XM and XMH groups. The percentage increase in the fiber CSA from 1 to 2 weeks after CTX injection was significantly greater in the XMH group than in the X group ($P<0.05$, Figure 6).

**Discussion**

In this study, we assessed the effects of treatment with MENS plus HBO in comparison to the effects of no treatment, MENS alone, or HBO alone on injured mouse TA muscle. Two weeks after the CTX injection, both the mean fiber CSA and the percentage mean fiber CSA increase were significantly greater in the group of mice which underwent combination MENS and HBO therapy than in the group of untreated mice. The fiber CSA did increase under sole treatment with MENS or HBO and even in the
absence of treatment, but the values did not differ significantly between these 3 post-CTX injection strategies.

**Effects of MENS alone**

According to our study data, treatment with MENS alone, in comparison to absence of treatment, did not have a significant effect on muscle wet weight, relative muscle wet weight, mean fiber CSA, or the percentage increase in fiber CSA over the 2 weeks after CTX injection. This result was consistent with that of a recent study in which MENS did not have a significant effect on the relative muscle weight or muscle protein content of injured TA muscle during the first 2 weeks after CTX injection\(^2\). In that study, however, MENS treatment, in comparison to no treatment, resulted in a significantly higher mean fiber CSA 2 and 3 weeks after the injection\(^2\). In another reported study, MENS appeared to have a beneficial effect on TA muscle wet weight and fiber CSA
measured 1 week after CTX injection. In our study, the mean fiber CSA and percentage increase in fiber CSA after MENS treatment were 123% and 118% greater, respectively, than the values obtained after absence of treatment. However, although the effect of MENS on injured skeletal muscle was evident, we do not have a clear explanation for it.

Although MENS seemed to enhance the muscle repair process, the effect on regeneration of injured muscle was relatively weak and insignificant. MENS may stimulate both the proliferative potential of muscle satellite cells and protein synthesis in injured skeletal muscle. Additional studies are needed to address this question.

**Effects of HBO alone**

Also according to our study data, treatment with HBO alone, in comparison to absence of treatment, did not have a significant effect on muscle wet weight, relative muscle wet weight, mean fiber CSA, or the percentage increase in fiber CSA over the 2 weeks after CTX injection. This result was not consistent with results of previous studies. We have no clear explanation for the discrepancy between our results and those reported previously. In our study, the mean fiber CSA and the percentage increase in fiber CSA after HBO treatment were 120% and 119% greater, respectively, than those in the untreated group. Because the O\textsubscript{2} level drops in damaged tissue, the healing that is usually seen after HBO is attributed to the ability of HBO to increase the O\textsubscript{2} level. Furthermore, HBO stimulates muscle satellite cells, which play a crucial role in the regeneration of skeletal muscle.
**MENS and HBO in combination**

When we combined MENS and HBO, we observed a marked increase in the mean fiber CSA and percentage increase in fiber CSA 2 weeks after CTX injection in comparison to values obtained in the total absence of treatment. We have no detailed explanation for beneficial effects of the combination therapy, it appears to us that the combination therapy enhances the regeneration of skeletal muscle promoted by MENS or HBO alone. Additional investigations are needed to elucidate precise mechanisms of the combination therapy with MENS and HBO in future.

**Conclusion**

Our study was the first to investigate the effect of combination MENS and HBO therapy on the regeneration of injured skeletal muscle. Combination MENS plus HBO therapy, in comparison to no-treatment or sole treatment with MENS or HBO alone, enhanced restoration of the fiber CSA. Our results are encouraging, and we anticipate future investigations that will elucidate the precise mechanisms underlying the benefit conferred by combination MENS and HBO therapy. Clinically, the combination MENS and HBO might facilitate the regeneration of injured skeletal muscle.

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