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The Efficacy of Microcurrent Therapy on Eccentric Contraction-Induced Muscle Damage in Rat Fast-Twitch Skeletal Muscle

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Abstract

Microcurrent (MC) therapy, in which a very small electric current is applied to the body, has widely been used to promote tissue healing and relieve symptoms. The aim of this study was to examine the effect of MC treatment on eccentric contraction (ECC)-induced muscle damage in rat fast-twitch skeletal muscles. Tibialis anterior muscles underwent 200 repeated ECCs *in situ* and were then stimulated (25 μA, 0.3 Hz) for 20 min (MC treatment). MC treatment was performed immediately after ECC and during a recovery period of 3 days (a total of 4 times). Three days after ECC, the muscles were excised and used for measure of force output and for biochemical analyses. In MC-treated muscles, tetanic forces at 20 Hz and 100 Hz were partially and fully restored, respectively, whereas in non-treated muscles, both forces remained depressed. Biochemical analyses revealed that MC treatment partially or completely inhibited ECC-induced reductions: in 1) the Ca^{2+}-release function of sarcoplasmic reticulum (SR), 2) proteolysis of ryanodine receptor, a Ca^{2+} release channel of SR, and 3) myosin ATPase activity. On the other hand, MC treatment was unable to lessen increases in the activity of calpain, a cytosolic, Ca^{2+}-activated neutral protease. These results indicate that MC treatment results in beneficial effects, such as restoration of muscle performance following ECC, although the precise mechanisms are still unknown at this time.

Keywords

Ryanodine Receptor, Sarcoplasmic Reticulum, SERCA, Myosin ATPase

1. Introduction

Eccentric contractions (ECCs), in which skeletal muscles are stretched while contracting, are a part of normal activities such as walking downstairs or lowing a heavy weight, and frequently cause an immediate and protracted loss of skeletal muscle force [1] [2]. The loss of muscle force is primarily ascribable to muscle damage, including increased membrane permeability [3], ultrastructural disruption [4], inflammation [5] and proteolysis [6]. It is well known that ECC is also responsible for delayed onset of muscle soreness (DOMS) that occurs secondarily to inflammation of muscle membranes [7].

Calpains are cytosolic, Ca^{2+}-activated neutral proteases and skeletal muscles contain the ubiquitous calpains (calpain-1 and calpain-2) and the muscle-specific calpain (calpain-3) [8]. There is evidence to suggest that ECC-induced proteolysis is mainly caused by the action of calpains [6] [9]. A ubiquitin-related proteasome, another cytosolic protease, is also activated in skeletal muscles subjected to ECC [1], although it is unclear to what extent muscle proteins are degraded by activated proteasome. Our laboratory and others have observed degradation of various proteins (e.g., actin, junctophilin, and dihydropyridine receptor) involved in excitation-contraction coupling days after ECC [2] [10]. It seems quite plausible that these changes account for prolonged force deficit.

Over recent decades, microcurrent (MC) therapy, which involves application of a very small electric current to the body, has demonstrated considerable potential for treatment of several forms of tissue damage. It has been shown that MC therapy can alleviate symptoms of tissue damage and promote tissue repair [11] [12] and is also effective in the cases that are recalcitrant to other forms of treatment [13]. However, there is very little information on whether MC therapy also has a beneficial effect on ECC-related muscle damage. To our knowledge, only two investigations into this issue have been performed. However, the results are controversial. One study showed that MC therapy that was given after ECC was unable to relieve DOMS induced in human skeletal muscles [7], whereas another study reported the beneficial effect on DOMS [14]. With the consideration that MC treatment has the potential to alleviate tissue damage, it might be expected that MC treatment would inhibit proteolysis of key proteins in excitation-contraction coupling that occurs with ECC. However, no studies have examined this point to date.

In light of these findings, we decided to elucidate the effect of MC treatment on ECC-induced muscle damage in rat fast-twitch skeletal muscles. This study focused on alterations in two proteins of sarcoplasmic reticulum (SR), *i.e.*, SR Ca^{2+}-ATPase (SERCA) and ryanodine receptor (RyR), and myosin ATPase, be-

cause the function of these three proteins is vital for skeletal muscle contraction [15] [16] [17]. In this study, we tested the hypothesis that MC treatment would facilitate force recovery following ECC by relieving depressions in the function of the three proteins investigated. The present experiments conducted with *in situ* ECC partially support this hypothesis.

2. Methods

2.1. Animals

All experimental procedures used in this study were approved by the Animal Care Committee of Hiroshima University. The experiments were performed on 9- to 10-week-old male Wistar rats (n = 16). The animals were individually housed in a cage in a thermally controlled room at 20˚C - 24˚C with a 12-h light/dark cycle and were provided with rat chow and water ad libitum. At the end of experiments, the rats were euthanized with an overdose of pentobarbital sodium (200 mg/kg body wt) followed by cervical dislocation.

2.2. Experimental Design

Two hundred ECCs (1-s train of 1-ms pulse at 50 Hz and 150˚ angular movement at $150^{\circ}\cdot s^{-1}$) were performed via electrodes to the peroneal nerve of the left hindlimb as described in detail previously [10]. After ECC, the animals were randomly divided into a MC-treated and a non-treated group (n = 8 for each group) Under anesthesia, with the use of an intraperitoneal injection of pentobarbital sodium (6 mg/100 g body wt), the epilation was done on the upper skin of anterior crural muscles from MC-treated rats. Two electrodes were then placed on the distal anterior side of the knee joint and the anterior proximal side of ankle joint, respectively and the anterior crural muscle of the left leg were stimulated (25 μA, 0.3 Hz) for 20 min (MC treatment), using an electrical stimulator (Elesas, Sunmedical, Japan). Non-treated rats were also anesthetized without MC treatment. These treatments for MC-treated and non-treated rats were performed immediately, 24 h, 48 h and 72 h after ECC (a total of 4 times).

2.3. Isometric Force Output

Two h after the last MC treatment, contracted (left leg) and rested (right leg) tibialis anterior (TA) muscles were excised under anesthesia (see above). Isolated TA muscles were mounted vertically in a stimulation chamber (30˚C) and allowed to rest for 10 min in standard Tyrode solution. Tetanic contractions were elicited by direct stimulation at various frequencies (1 - 100 Hz) using supramaximal voltage, 1-ms pulses, and trains of 1.5 s. Forces were recorded on a personal computer, analyzed using dedicated software (LabChart, ADInstruments, Japan).

2.4. SR Ca^{2+}-Uptake and Release Rates

Muscle pieces were homogenized in 9 volumes ($vol\cdot mass^{-1}$) of the ice-cold buffer

(pH 7.4) consisting of 300 mM sucrose, 20 mM MOPS, 0.83 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 2.2 μM leupeptin and 1.4 μM pepstatin A. The protein content of the homogenate was determined by the Bradford assay using bovine serum albumin as the standard [18]. SR Ca^{2+}-uptake and release rates were measured using the Ca^{2+} fluorescent dye indo-1 as previously described [19]. Briefly, aliquots of the homogenate were incubated for 3 min at 37˚C in the assay buffer (pH 7.0) composed of 100 mM KCl, 20 *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid, 10 mM NaN_3, 6.8 mM potassium oxalate, 0.5 mM $MgCl_2$ and 1 μM indo-1. SR Ca^{2+} uptake was initiated by adding 1 mM Mg-ATP and allowed to continue until little or no change in the Ca^{2+} concentration ($[Ca^{2+}]$) was observed. Ca^{2+} release was then initiated by adding 10 mM 4-chloro-m-cresol. The $[Ca^{2+}]$ was monitored using a fluorometer (CAF-110, Nihon-Bunko, Japan) and was computed according to the ratiometric method [20].

2.5. Western Blot

Western blot was performed using the following primary antibodies: anti-RyR 1/2 (1:2500 dilution; Thermo Scientific, MA3-911), anti-SERCA1a (1:5000 dilution; Thermo Scientific, MA3-925), anti-glyceraldehyde-3-phophate dehydrogenase (GAPDH; 1:5000 dilution; Wako, 019-25471). Aliquots of the homogenate prepared for calpain activity experiment (see below) were diluted with sodium dodecyl sulfate (SDS)-sample buffer consisting of 62.5 mM Tris/HCl (pH 6.8), 10% ($vol \cdot vol^{-1}$) glycerol, 5% ($vol \cdot vol^{-1}$) 2-mercaptoethanol, 2% ($mass \cdot vol^{-1}$) SDS and 0.02% ($mass \cdot vol^{-1}$) bromophenol blue. Twenty micrograms of protein were applied to a 7% ($mass \cdot vol^{-1}$) polyacrylamide gel and SDS-polyacrylamide gel electrophoresis was run at 100 V for 2 h at room temperature. The separated proteins were then transferred onto polyvinylidene difluoride membranes using a semi-dry transfer system (2 mA/cm^2, 75 min). The membranes were blocked with phosphate-buffered saline containing 3% ($mass \cdot vol^{-1}$) skim milk and 0.1% ($vol \cdot vol^{-1}$) Tween-20 for 1 h at room temperature, followed by overnight incubation with primary antibody at 4˚C. The membranes were then incubated with secondary antibody (1:5000 dilution; Dako, P0260) for 1 h at room temperature. Immunoreactive bands were visualized with chemiluminescence reagent (GE Healthcare, USA) and evaluated using Image J software (National Institutes of Health, USA). In addition to the experimental samples, each blot always contained the standard sample. Densitometrically evaluated amounts of RyR and SERCA1 were normalized by reference to those in the standard sample. Equal loading of proteins was monitored by the band density of GAPDH.

2.6. Myosin ATPase activity

Myofibril extracts were prepared by the methods of Tsika *et al.* [21]. Muscle pieces were homogenized in 10 volumes ($vol \cdot mass^{-1}$) of the ice-cold buffer (pH 6.8) composed of 250 mM sucrose, 100 mM KCl, 20 mM imidazole and 5 mM

EDTA. After centrifugation at 1000 *g* for 10 min at 4˚C, the supernatant was discarded. The resulting pellet was rehomogenized in 10 volumes (vol·mass^{-1}) of 175 mM KCl (pH 6.8) containing 0.5% (vol·vol^{-1}) Triton X-100. This homogenizing-centrifugation cycle was repeated three times. The homogenizing-centrifugation cycle was then repeated two more times using a solution (solution 1) consisting of 150 mM KCl and 20 mM imidazole (pH 7.0). The resulting pellet was suspended in solution 1. The protein content of myofibril extraction was determined in a manner similar to that described above.

Myosin ATPase activity was spectrophotometrically determined in myofibril extracts at 37˚C [22]. The reaction mixture was composed of 30 mM KCl, 30 mM Tris/HCl (pH 7.0), 2 mM sodium azide, 1 mM $MgSO_4$, 1 mM EGTA, 1.1 mM $CaCl_2$, 0.4 mM NADH, 10 mM phosphoenolpyruvate, 18 U·ml^{-1} pyruvate kinase and 18 U·ml^{-1} lactate dehydrogenase. The reaction was started by adding ATP to give a final concentration of 1 mM. The oxidation of NADH was monitored in a spectrophotometer (V-530, Jasco, Japan) for 3 min (340 nm). Myosin ATPase activity was calculated as micromoles per minute per milligram myofibrillar protein.

2.7. Calpain Activity

Immediately after measurement of force output, muscle pieces were homogenized on ice in 9 volumes (vol·mass^{-1}) of 20 mM Tris buffer (pH 7.4) containing 5 mM EDTA, 5 mM EGTA, 1 mM dithiothreitol, 0.5 mM PMSF, 14 μM pepstatin A and 10 μg/ml 4-(2-aminoethyl)-benzenesulfonyfluoride (AEBF). Maximal calpain activity was measured as previously described [22]. Briefly, aliquots of the homogenate were incubated for 5 min at 37˚C in the assay buffer (pH7.4) consisting of 20 mM Tris, 5 mM $CaCl_2$, 1 mM dithiothreitol, 14 μM pepstain A and 10 μg/ml AEBF. The reaction was started by adding 125 μM N-Succinyl-Leu-Tyr-7-amido-4-methylcoumarin (SLY-AMC). Fluorescence of the liberated AMC was monitored with a fluorometer (RF-5000, Shimazu, Japan) for 7 min (excitation 380 nm, emission 460 nm). The protein content of the homogenate was determined in a manner similar to that described above.

2.8. Statistics

Statistical analyses were conducted with Sigma-Plot statistical software (version 12, Systat Software, USA). All data are presented as means ± SE. The effects of ECC alone and ECC+MC treatment were investigated using a one-way ANOVA. When significant differences were detected, Holm-Sidak post hoc test was performed. The acceptable level of significance was set at $P < 0.05$.

3. Results

3.1. Isometric Force Output

In our preliminary experiment, we observed that MC treatment alone exerted little or no effect on force, SR Ca^{2+}-handling function, SERCA and RyR amounts

or myosin ATPase and calpain activities. In both non-treated and MC-treated rats, ECC brought about depressions in tetanic force at 20 Hz, but the extent of the reductions was greater in the former (**Figure 1(a)**). Force in ECC muscles amounted to 46% and 77% of that in rested muscles from non-treated and MC-treated rats, respectively. In non-treated rats, force at 100 Hz decreased in ECC muscles to 78% of that in rested muscles (**Figure 1(b)**). On the other hand, in MC-treated rats, no significant differences were observed between rested and ECC muscles. Prolonged low-frequency force depression (also referred to as low-frequency fatigue) is characterized by a greater loss of force at low frequencies of stimulation than that at high frequencies [17] [23]. Our results of force indicate that an *in situ* ECC model utilized in this study can elicit this type of muscle fatigue.

3.2. SR Ca^{2+}-Handling Function

In both non-treated and MC-treated rats, ECC had no influence on SR Ca^{2+}-uptake rate (**Figure 2(a)**). On the other hand, ECC decreased SR Ca^{2+}-release rate to 74% and 87% of that in rested muscles from non-treated and MC-treated rats, respectively (**Figure 2(b)**). The release rate was significantly greater in ECC muscles from MC-treated rats than in those from non-treated rats.

3.3. Amounts of SERCA and RyR

Changes in the amounts of SERCA and RyR were in agreement with those of SR

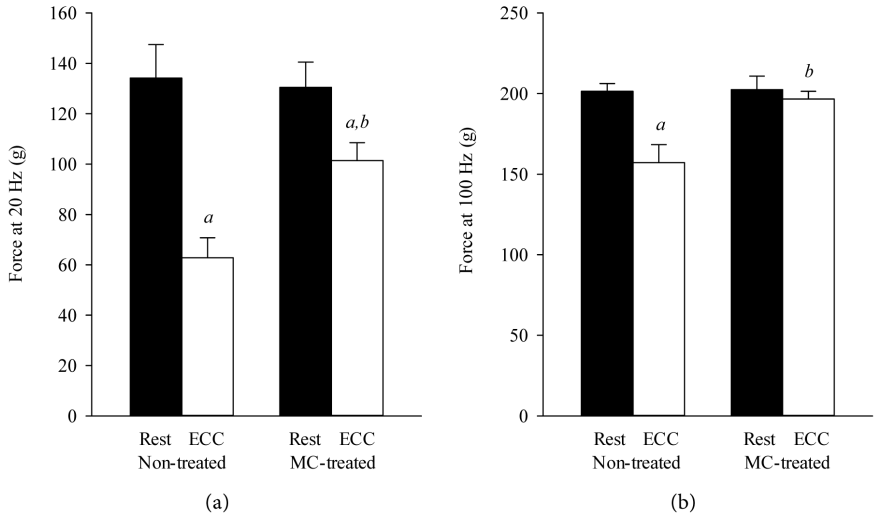

Figure 1. Effects of microcurrent (MC) treatment and eccentric contraction (ECC) on force output. ECCs were repeated in the anterior muscles of the left hindlimb for 200 cycles. The rested muscles of the contralateral (right) legs were used as a control. MC treatment was applied to the contracted (ECC) muscles from MC-treated rats 4 times in total, once a day. Three days after ECC, tibialis anterior muscles were excised and used for the experiment. Tetanic contractions of isolated tibialis anterior muscles were evoked by electrical stimulation at 20 Hz (a) and 100 Hz (b). Values are means ± SE (n = 8 for each group). $^{a}P < 0.05$, significantly different from rested muscles within rats. $^{b}P < 0.05$, significantly different from ECC muscles from non-treated rats.

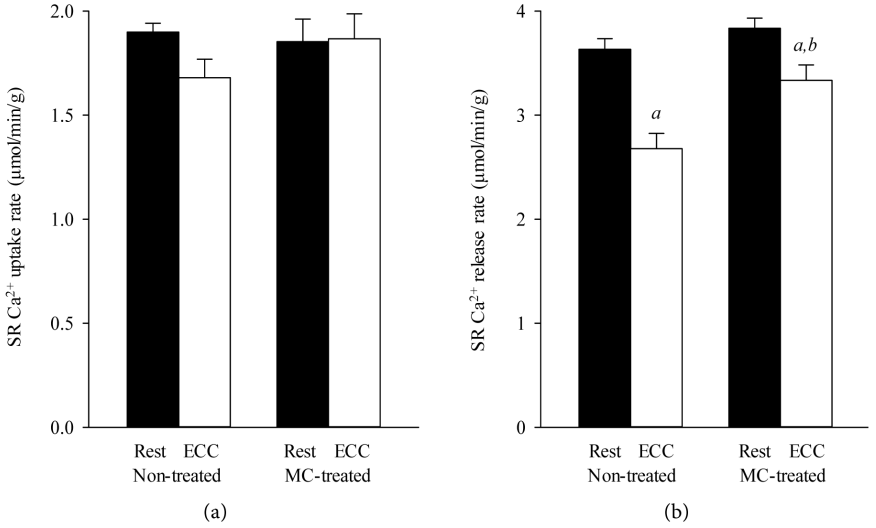

Figure 2. Effects of microcurrent (MC) treatment and eccentric contraction (ECC) on sarcoplasmic reticulum (SR) Ca^{2+}-uptake (a) and release (b) rate. For the protocols of treatment with MC and ECC, see legend of **Figure 1**. Values are means ± SE (n = 8 for each group). $^{a}P < 0.05$, significantly different from rested muscles within rats. $^{b}P < 0.05$, significantly different from ECC muscles from non-treated rats.

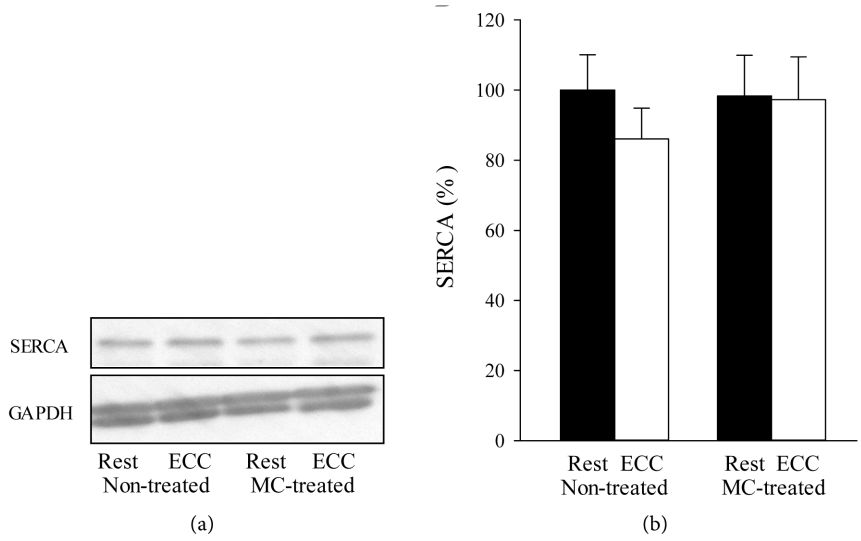

Figure 3. Effects of microcurrent (MC) treatment and eccentric contraction (ECC) on sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) amount. For the protocols of treatment with MC and ECC, see legend of **Figure 1**. (a) immunoblot analysis of SERCA. Equal loading of proteins was monitored by the band density of glyceraldehyde 3-phosphate dehydrogenase (GAPDH); (b) means ± SE (n = 8 for each group) of SERCA amount. The results were expressed as percentages of the values in rested muscles from non-treated rats.

Ca^{2+}-handling function. No changes in the SERCA amounts were observed in ECC muscles from non-treated and MC-treated rats (**Figure 3**). Consistent with the previous findings [22], in addition to full-length RyR, several bands that migrate faster were found in both rested and ECC muscles (**Figure 4(a)**) and most

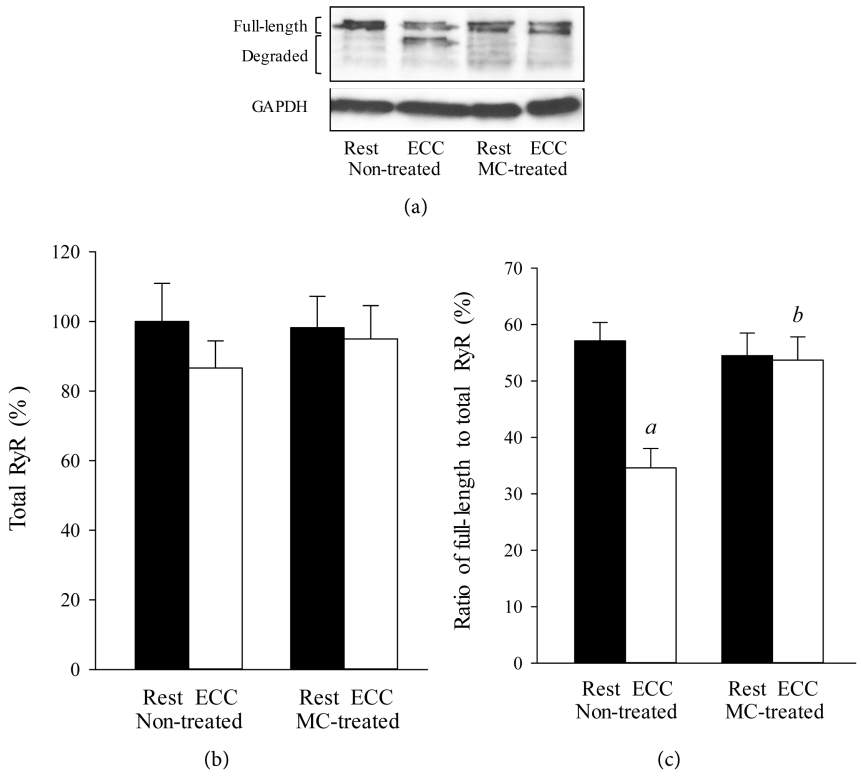

Figure 4. Effects of microcurrent (MC) treatment and eccentric contraction (ECC) on ryanodine receptor (RyR) amount. For the protocols of treatment with MC and ECC, see legend of **Figure 1**. (a) immunoblot analysis of RyR. In all muscles, in addition to full-length RyR, several bands that migrate faster were found and most likely corresponds to degraded RyR. ECC muscles from non-treated rats displayed the relatively larger amounts of degraded RyR. Equal loading of proteins was monitored by the band density of glyceraldehyde 3-phosphate dehydrogenase (GAPDH); (b) means ± SE (n = 8 for each group) of total RyR amount. The results were expressed as percentages of the values in rested muscles from non-treated rats; (c) mean ± SE (n = 8 for each group) of the ratio of full-length to total RyR (degraded + full-length). $^{a}P < 0.05$, significantly different from rested muscles within rats. $^{b}P < 0.05$, significantly different from ECC muscles from non-treated rats.

likely correspond to degraded RyR [10] [24]. ECC-induced reductions in the ratio of full-length to total RyR (*i.e.*, increases in degraded RyR) were observed only in non-treated rats (**Figure 4(c)**).

3.4. Myosin ATPase and Calpain Activities

ECC decreased myosin ATPase activity to 76% and 88% of that in rested muscles from non-treated and MC-treated rats, respectively (**Figure 5**). However, significant differences between rested and ECC muscles were found only in non-treated rats. To determine whether the beneficial effects observed of MC treatment are mediated through changes in calpain activity, measurements of calpain activity were performed in the present study. As shown in **Figure 6**, ECC significantly increased the activity to 161% and 147% of that in rested muscles from non-treated and MC-treated rats, respectively. Although in ECC muscles, there

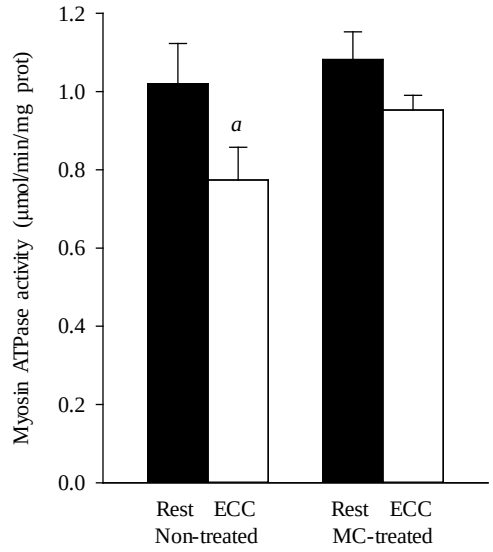

Figure 5. Effects of microcurrent (MC) treatment and eccentric contraction (ECC) on myosin ATPase activity. For the protocols of treatment with MC and ECC, see legend of **Figure 1**. Values are means ± SE (n = 8 for each group). [a]$P < 0.05$, significantly different from rested muscles within rats

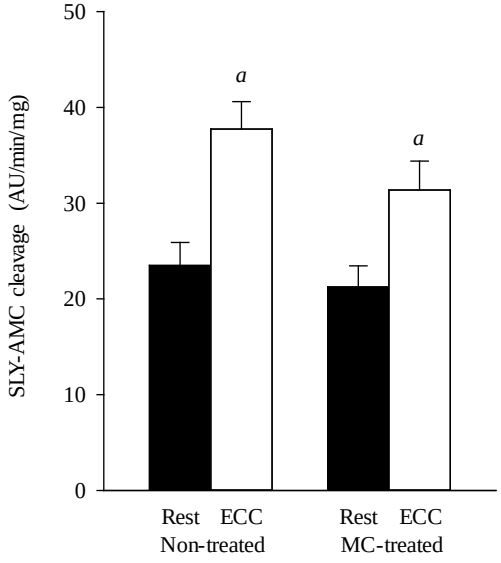

Figure 6. Effects of microcurrent (MC) treatment and eccentric contraction (ECC) on calpain activity. For the protocols of treatment with MC and ECC, see legend of **Figure 1**. Values are means ± SE (n = 8 for each group). [a]$P < 0.05$, significantly different from rested muscles within rats. SLY-AMC, N-succinyl-Leu-Tyr-7-amido-4-methylcoumarin; AU, arbitrary units.

was a trend for a lower activity in MC-treated than in non-treated rats, these differences did not reach significance level.

4. Discussion

As reported many times previously, features common to ECC are that this type

of muscle contraction results in greater depressions in force, compared to concentric and isometric contractions and that restoration of force is a very slow process, in some instance requiring several days or more for full recovery [22] [25]. The present investigation, for the first time, provides evidence that MC treatment can facilitate restoration of force production after ECC. Three days after ECC, force remained depressed in non-treated muscles, while forces at 20 Hz and 100 Hz were partially and fully restored in MC-treated muscles, respectively.

In rat fast-twitch muscles, force steeply rises as stimulation frequency increases in the range of 20 - 60 Hz and it levels off at higher frequencies [26]. It has been accepted that SR Ca^{2+} release is one of the determinants of submaximal force (*i.e.*, force at 20 Hz) [17] [23] and that maximal specific force (*i.e.*, force at 100 Hz) correlates with myosin ATPase [26] [27]. These findings suggest that for forces at 100 Hz and 20 Hz, promotion of force recovery with MC treatment may involve a maintenance of myosin ATPase activity (**Figure 5**) and a blunting of depressions in SR Ca^{2+}-release function (**Figure 2B**), respectively.

Our results point out that the blunting of depressions in SR Ca^{2+}-release function is, at least partly, due to inhibited proteolysis of RyR (**Figure 2** and **Figure 4**). Accumulating evidence reveals that ECC triggers extracellular Ca^{2+} to enter the muscle cells and that an elevation of cytoplasmic Ca^{2+} concentration due to Ca^{2+} entry activates the calpains [28] [29]. Given that removal of extracellular Ca^{2+} or application of a calpain inhibitor can attenuate ECC-dependent proteolysis of various proteins [26], it seems quite plausible that proteolysis with ECC is ascribable mainly to activated calpains. However, in contrast to these previous findings, the present results regarding calpain activity indicate that the inhibitory effect of MC treatment on RyR proteolysis does not lie in the inhibition of calpain activation (**Figure 6**). These agree with a very recent study showing that application of a calpain inhibitor is unable to prevent ECC-induced RyR proteolysis [10]. The mechanisms underlying ECC-elicited RyR proteolysis are unclear, but as pointed out by Kanzaki *et al.* [10], one likely possibility is an involvement of proteasome. Ionsitol 1,4,5-triphosphate receptor is a protein that structurally and functionally resembles RyR [30]. The fact that the proteasome can degrade this receptor makes it likely that the proteasome may fulfill a vital role in ECC-induced RyR proteolysis. If such is the case, MC treatment would mitigate the proteasome activation that occurs with ECC. This issue is an important subject for further research.

The mechanisms for changes in myosin ATPase activity with ECC and MC treatment are also equivocal. Our previous study, using the same model for ECC as in this study [22], has demonstrated that up to 4 days after ECC, ECC-related reductions in myosin ATPase activity are not accompanied by proteolysis of myosin heavy chain where myosin ATPase is situated. It has been shown that the concentration of nitric oxide increases in the muscle cell for several days after ECC [31] and that increased nitric oxide adversely affects the contractile proper-

ties via *S*-nitrosylation and/or nitration of various proteins [32] [33]. The ECC-induced reduction in myosin ATPase activity presented here might be expected to stem from structural perturbations in myosin heavy chain, which are mediated through the action of nitric oxide.

Muscle damage and resultant depressions in muscle performance are universal symptoms familiar to most athletes, because muscle contractions during many of sport activities and training include a substantial eccentric component. To date, various treatment strategies have been done to attenuate the extent of the depression in muscle function and to facilitate recovery. These include static stretch [34], nutritional supplement [35], massage therapy [36] and cryotherapy (*i.e.*, cooling the exercised limbs) [37]. Little scientific evidence, however, exists to support the effectiveness of any of these interventions. This study provides evidence that MC therapy results in beneficial effects, such as restoration of muscle performance following ECC, although the precise mechanisms are still unknown at this time.

Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

Author Contributions

Y.H. and M.W. designed the research; Y.H., D.W. and C.A. performed experiments; Y.H., D.W. and C.A analyzed data; Y.H., D.W., K.K and M.W. interpreted results of experiments; Y.H and M.W. prepared figures; Y.H. and M.W. drafted manuscript; Y.H., S.M. and M.W. edited and revised manuscript; all authors approved final version of manuscript. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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