ABSTRACT

The Effects of Application Timing for Electromyostimulation on Denervated Skeletal Muscle Atrophy

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Introduction: Electromyostimulation (EMS) has been widely utilized to reduce muscle atrophy in clinical setting. Nevertheless, the effects of EMS on denervation atrophy and the optimal timing for the initiation of EMS remain unclear.

Objectives: This study was performed to evaluate the effects of EMS on denervated muscles in relation to the changes in myosin heavy chain (MHC) isoform, and to determine the optimal timing for EMS application.

Methods: A total of 18 Sprague-Dawley rats were divided into four groups. Two treatment groups underwent a sciatic nerve crush and received EMS for

30 minutes a day, 5 days a week, for 2 weeks. EMS was initiated on post-injury day 1 for immediate EMS (IES, n=5) group and on post-injury day 15 for delayed EMS (DES, n=5) group. The denervation control (Dcon, n=4) group received identical operation without EMS. Rats in the normal control (Ncon, n=4) group did not receive either surgery or EMS. Foot print analysis using the CatWalk method, extensor postural thrust test and nerve conduction studies were also performed to assess the denervation and reinnervation process after injury. Four weeks after injury, muscle wet weight and cross-sectional area of muscle fiber were measured. MHC isoform types was analyzed in both protein homogenates and single muscle fibers.

Results: Significantly lower IIx isoform and higher proportion of MHC IIb isoform in gastrocnemius muscle were observed in IES group than in Dcon group, which was not found in DES group. IES group also showed larger cross-sectional area of denervated gastrocnemius muscle fiber and lower hybrid single fiber proportion compared with DES group.

Conclusions: These results indicate that immediate EMS is more effective in recovery of denervation induced MHC changes compared to the delayed EMS.

Keywords: electromyostimulation, muscle atrophy, myosin heavy chain, denervation

Student number: 2010-30561

CONTENTS

LIST OF FIGURES

INTRODUCTION

Skeletal muscle atrophy is a change attributed to the conditions of immobilization, denervation, muscle unloading, aging, starvation, cancer and other systemic diseases. Skeletal muscle atrophy is characterized by decreases in protein content, fiber diameter, and production of muscle strength, and resistance to fatigue. Denervation is the loss of nerve supply due to diseases or injury of peripheral nerve or motor neurons. Skeletal muscles after denervation undergo no contractile activity, rapid loss of muscle mass, muscle fiber atrophy, as well as impaired function (1-3).

The adaptive property of the muscle in response to the imposed functional demands is called muscle plasticity, which involves changes in the quantity or types of protein. Myosin has been regarded as a cellular “marker” for muscle plasticity in response to the new environmental requirement (1). Myosin is an important structural and regulatory protein that constitutes the contractile apparatus of skeletal muscle, and very sensitive to the degree of mechanical stress imposed on the muscle. The quantity and the isoform type of myosin heavy chain (MHC) are altered in response to mechanical stress.

MHC is expressed as different isoforms with distinct functional properties, thereby contributing to functional diversity of the muscle. In adult mammalian skeletal muscles, at least four different isoforms of MHC are expressed: I, IIa, IIx and IIb, corresponding to fiber type I, IIA, IIX, and IIB, respectively. Mammalian skeletal muscle fibers can be categorized into slow and fast twitch types based on contractile properties, which, in part, vary depending on differences in the MHC isoforms (4, 5). The maximal

contractile velocities of rodent muscle fibers are known to be slowest in type I followed by IIA, IIX, and IIB (6-10). Slow-twitch (type I) fibers are known to have low adenosine triphosphatase (ATPase) activity and high oxidative enzyme capacity, which are designed for antigravity and sustained locomotor activities. Fast-twitch glycolytic (type IIA, IIX, and IIB) fibers are characterized by high ATPase activity, high glycolytic enzyme activity, and specialized for short duration and high power output activities such as sprinting. The distribution of the different types of fibers depends on individual muscles. Slow type I fibers are abundant in postural muscle such as soleus, whereas type II fibers are more widely distributed in fast type muscles such as extensor digitorum longus.

A fiber containing only one MHC isoform is referred to as a pure fiber, whereas a fiber that expresses more than one MHC isoform is referred to as a hybrid fiber. The proportion of the hybrid fibers in normal skeletal muscles is typically low, but their numbers could be dramatically increased under conditions of MHC and fiber type transformation. It is widely accepted that the proportion of hybrid fibers is higher in transitional state from one ‘steady–state’ to another than in normal muscle (11-13).

Denervation-induced muscle atrophy leads to adaptation of MHC, where MHC content decreases (14) and the proportion of MHC isoforms changes (14-17). Conversion from slow to fast-twitch fiber has been reported in animals (18, 19) as well as in humans (20-22). Hybrid fibers exhibiting multiple MHC isoforms markedly increased in the denervated muscles (17, 23).

Physical therapy interventions such as stretching, strengthening and endurance exercises, and electrical stimulation have been used to prevent the atrophy of denervated muscle until the reinnervation occurs (24-27). These interventions are designed to overcome non-physiologic use, overuse, or disuse by early mobilization. In addition, such therapies may broaden the therapeutic window of time for functional restoration (28). Muscle atrophy after denervation is usually combined with disuse and degeneration (1, 29). Immobilization leads to degenerative changes, but these changes may be reversible within a short period of time. However, prolonged denervation and atrophy causes irreversible changes in the muscle with no hope for functional recovery, especially when growing nerve fibers have to cover long distances to reconnect to their target muscles (30). Therefore, early mobilization should not be overlooked.

One of the therapeutic approaches generally used for the treatment of denervated muscle is electromyostimulation (EMS). EMS is direct muscle stimulation that leads to muscle contraction (27, 31). The main purpose of EMS in denervated muscles is to prevent or reverse atrophic changes and provide a substitute for innervation before reinnervation (32). Even though the benefits of EMS in patient and animal models are still controversial, EMS has been successfully translated into the clinical practice (33, 34).

The rationale for the application of EMS in denervated muscle rests on the notion that muscle activity, not neurotrophic factors, is the most important factor for the regulation of muscle fiber properties (32). Thus, EMS has been considered as a useful tool to delay muscle atrophy and preserve the

normal properties of denervated muscle. Kern et al. demonstrated the increase of the surviving fibers in size and regeneration of new myofibers following EMS in human long-term denervated muscles (35). Eberstein et al. reported that EMS applied before reinnervation could preserve the structure of the endplate as well as accelerate recovery of normal function in reinnervated muscle fibers (27). Lim et al. reported that the upregulated expression of apoptosis-related factors was reduced in partially denervated muscles, and the extent of muscle atrophy was also reduced (36).

However, EMS has been reported as a potential inhibitor of axon terminal sprouting. Brown et al. reported that nerve axon terminal sprouting could be inhibited by EMS in partially denervated muscle (37). In severe denervated tibialis anterior muscles by avulsion of L4 spinal root, axon sprouting may be inhibited by non-physiologic neuromuscular activity in acute phase (38). One recent study showed that EMS could interfere with muscular excitability recovery leading to impaired functional recovery measured by the sciatic functional index and accentuate muscle fiber atrophy in a crushed sciatic nerve model (39).

Furthermore, optimal timing for EMS application remains also controversial. EMS immediately after nerve injury has generally been assumed to be most beneficial. As the interval between the onset of denervation and the start of stimulation increases, the recovery was inhibited (32). Andreose et al. reported that the degradation rate of slowly degrading acetylcholine receptors (AChRs) at the neuromuscular junction was accelerated after denervation, and they were replaced with rapidly degrading

AChRs (40). EMS applied in the initial phase after denervation were able to reverse the effect of denervation, while EMS initiated after the AChRs became destabilized could not.

The lack of consensus on the use of EMS in denervated muscles is associated with that a variety of stimulus parameters, onset time to stimulation, types of electrode, types of lesion and treatment regimens have been used in previous studies, which makes comparisons very difficult (27, 35-37, 39-42).

We have focused to the important but frequently neglected question about the effects of different onset times for EMS applied for denervated skeletal muscles. Therefore, the purpose of the present study was to find out the effects of EMS on muscle plasticity in the partially denervated rat skeletal muscles. We also aimed to establish the optimal initiation timing of EMS in the partial denervation model.

MATERIALS AND METHODS

1. Animals

A total of eighteen 8-week-old male Spraugue-Dawley rats (350-400 g) were used for the study. The rats were housed in pathogen-free conditions at ~20°C and exposed to a reverse light-darkness cycle (12:12 hr) each day. All the procedures of animal housing and surgical interventions were performed in accordance with the Canadian Council on Animal Care Guidelines under institutionally reviewed animal protocols.

2. Experimental Protocol

Before the nerve injury, all the rats were anesthetized with zoletil (zolazepam and tiletamine, 30 mg/kg) and xylazine (10 mg/kg) by intraperitoneal injection. The sciatic nerve trunk was exposed by a posterior thigh-split incision under a sterile condition.

The 18 rats were divided into 4 groups. Except the normal control group (n=4), three groups of rats (n=14) received partial denervation injury. For partial denervation, 10 mm distal to the sciatic notch of the sciatic nerve trunk was crushed with a non-serrated hemostatic forceps for 1.5 min (Fig. 1). To apply constant pressure to the nerve trunk, the compression force was controlled using the same level of locking forceps, and the inner margin of the nerve trunk was located at 3 mm proximal to the end of the jaws between the arms of the forceps (43). After the muscle and skin were closed, 10 mg/kg of gentamycin was injected into gluteus muscle.

Ten out of the 14 rats with partial denervation injury were assigned into EMS groups and received EMS on gastrocnemius muscle. Immediate EMS (IES) group (n=5) and delayed EMS (DES) group (n=5) received EMS on starting from day 1 to 14 and from day 15 to day 28, respectively. The remaining rats with partial denervation injury were assigned into the denervation control (Dcon) group (n=4). All the rats were euthanized and examined after 4 weeks of the injury. The rats in the normal control (Ncon) did not receive any intervention (n=4). The experimental protocol is summarized in Fig. 2.

3. Electromyostimulation (EMS)

With the rat in a prone position, both legs were tied to a fixation frame, and the stimulation electrodes were applied to the motor points of the gastrocnemius muscles. Using a portable electrostimulator (CEFAR Rehab 4 PRO, CefarCompex, Sweden), EMS was applied at the motor point of the right gastrocnemius muscle for 30 min/day, 5 days/week (Monday-Friday) for 2 weeks (Fig. 3). The stimulation parameters were as follows (36): 1-Hz frequency, 10-ms stimulation duration, 5-mA stimulus intensity, biphasic triangular impulses. To minimize the difference between the groups in the level and amount of activity after denervation, the rats in the Ncon and Dcon group were also placed in a prone position, and both legs were tied to the fixation frame for the same amount of time and number of sessions as the EMS-treated rats.

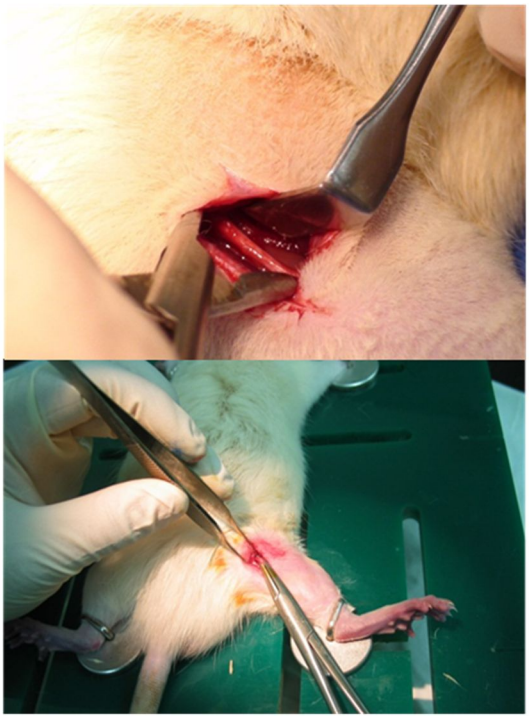

Figure 1. Partial denervation crush model by using non-serrated hemostatic forceps.

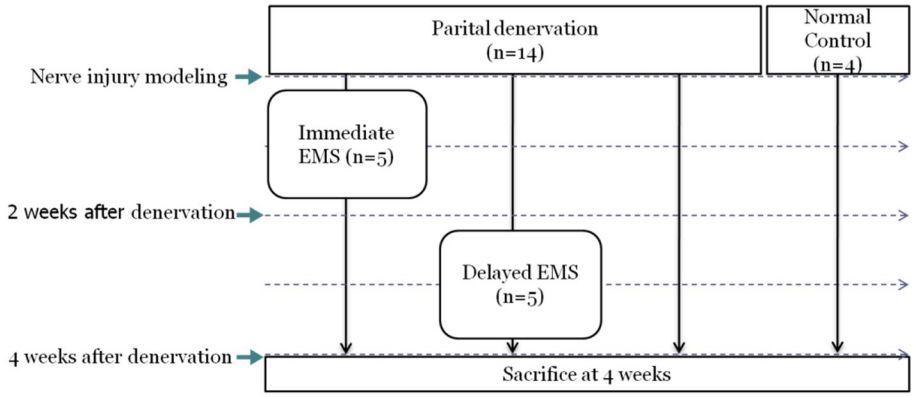

Figure 2. Overview of the experimental protocol.

Figure 3. Electromyostimulation in the denervated gastrocnemius muscles.

4. Outcome Evaluations

4.1. Foot print area

Analysis of gait was performed on walking rats using the CatWalk method (Fig. 4) (44-46). Briefly, light from a fluorescent tube was sent through a glass plate. Light rays were completely reflected internally. As soon as anything, e.g. a rat's paw, was in contact with the glass surface, light was reflected downwards, which resulted in a sharp image of a bright paw print (Fig. 5). The whole run was recorded via a camera placed under the glass plate.

The foot print area (expressed in cm^2) related to the affected single paw was measured. This parameter describes the total floor area contacted by the affected paw during the stance phase (47).

4.2. Extensor Postural Thrust (EPT) Test

The motor functional recovery of the rat model was examined by extensor postural thrust (EPT) test (48). The left hindlimb (unoperated side) and the tail were supported with the examiner's left hand while the torso was grasped with the right hand, so that the body weight was supported by the distal metatarsus and toes. As the body was lowered to the platform of a digital balance scale with an approximate range of 0–500 g, the rat extended its right hind limb (denervated limb) onto the surface of the balance and the extensor thrust was measured as the force in grams resisting the platform by the heel when the rat begins to bear weight on the scale (Fig. 6). The EPT was repetitively measured at least five times in each side, with 1-2 min rest

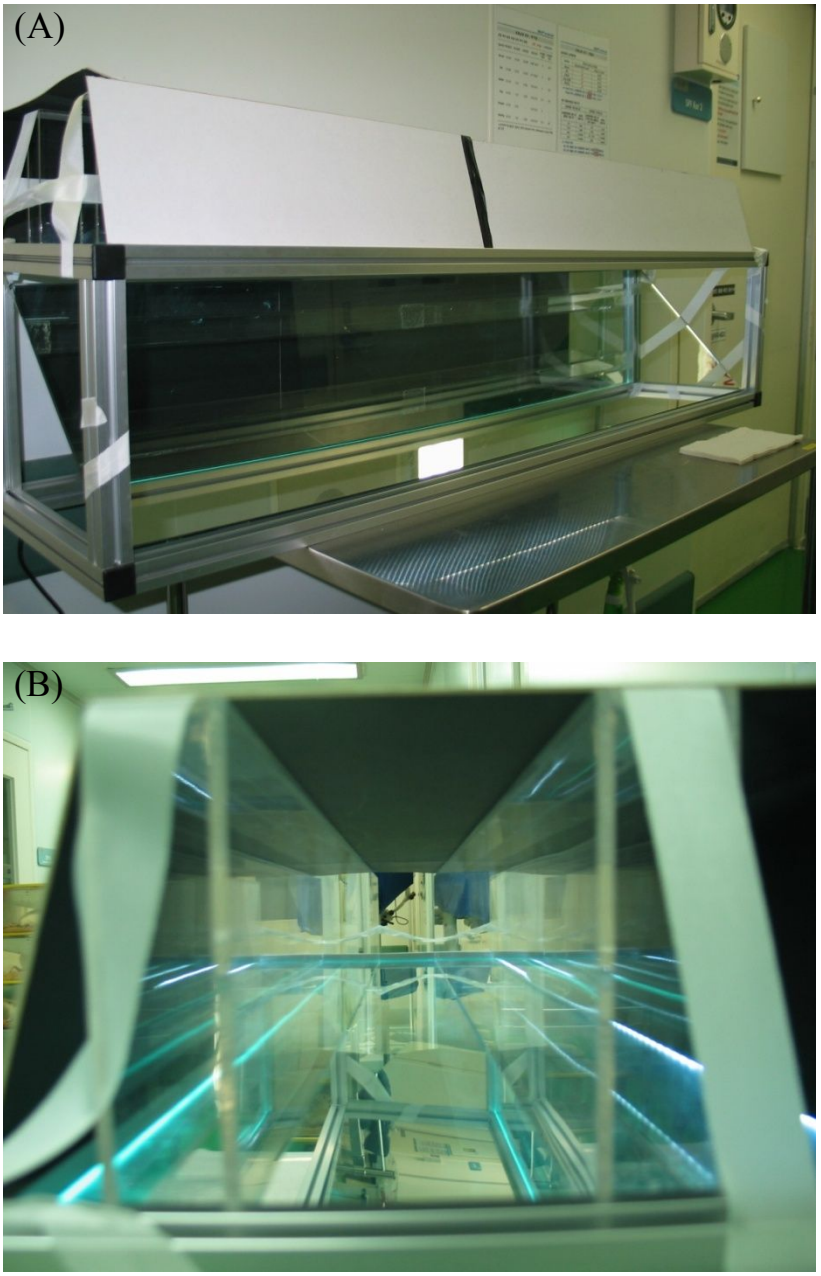

Figure 4. The experimental setup of the front view (A) and the side view (B) for the CatWalk method. The animals were allowed to traverse a walkway with a glass floor (163x34cm).

Figure 5. Image of bright paw prints. Foot print area by the affected paw during the stance phase was measured using the CatWalk method as the animals are intensively observed during the walkway crossing. Top: The floor monitored via a mirror placed at an angle of 45˚ under floor. Bottom: Example of Black/White image in the floor.

Figure 6. Extensor postural thrust test. The amount of weight borne on the denervated limb placed upon a digital scale was recorded.

between measurements. The same was performed the contralateral, unaffected limb. The measured values on the normal and denervated sides, were incorporated into a formula for calculating the percentage motor deficit (49):

$$\text{Percentage motor deficit (\%)} = (\text{NEPT} - \text{DEPT})/\text{NEPT} \times 100$$

, where NEPT is the value of the EPT on normal side and DEPT is the value of the EPT on denervated side.

4.3. Electrophysiologic Examinations

At 4 weeks after the partial denervation injury, the compound muscle action potentials (CMAPs) of the tibial nerve were measured before harvesting the muscles. We stimulated the tibial nerve at the popliteal fossa using a stimulator constructed in-house with a needle EP electrode (Fig. 7).

CMAPs were recorded from active and reference electrodes attached to the gastrocnemius muscles (filtering frequency 10 Hz to 10 kHz, sweep speed 1 ms/division, sensitivity 1 mV/division) using a portable two-channel electromyography/nerve conduction velocity system (Medelec Synergy; Oxford Instrument Medical Systems, Oxford, UK). The ratio of the amplitude of the injured side to the intact side was used as a main parameter (36).

4.4. Muscle Wet Weight Measurement

Whole gastrocnemius muscles were resected from just below the origin site of the gastrocnemius proximally to just above the calcaneal insertion of triceps surae distally in both legs. Thereafter, soleus muscles were taken apart from the whole triceps surae muscles by detaching the small longitudinal muscles

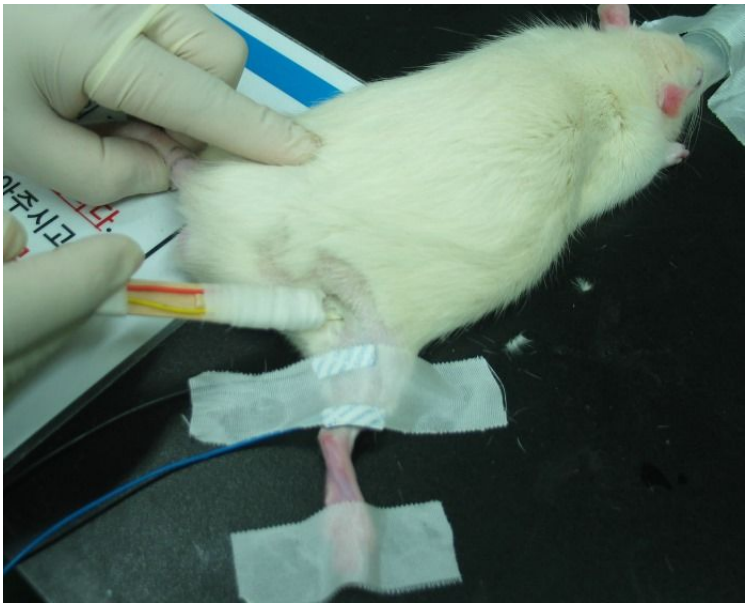

Figure 7. Electrophysiologic examination. CMAPs were evoked by electrical stimuli applied to the tibial nerve at the popliteal fossa using a stimulator constructed in-house with a needle EP electrode and recorded from the gastrocnemius muscles.

lying underneath the gastrocnemius. The wet weights of the gastrocnemius and soleus muscles were measured, and the ratio of denervated muscle wet weight to that of the intact side was calculated for each specimen.

4.5. Morphometric Analysis for Gastrocnemius muscle

A block of muscle taken from the mid-belly of gastrocnemius muscle was fixed in 4% paraformaldehyde. The fixed muscle specimens were washed with water, dehydrated in a graded ethanol series, cleared in xylene, embedded in paraffin and cut into 7-μm-thick sections. Haematoxylin & Eosin (H&E) staining was then applied to the sections, and images were observed under light microscopy. Photographs were taken from five fields (×200) chosen randomly in the central region of one cross-section of each gastrocnemius muscle with the aid of an AxioCam HR digital camera (Carl Zeiss, Göttingen, Germany). Images were digitalized into an Axiovision imaging software (Carl Zeiss, Göttingen, Germany), followed by analyses with a ImageJ 1.44a software (Wayne Rasband, National Institutes of Health, USA, http://rsb.knfo.ih.gove/ij) to measure the cross-sectional area (CSA) of the muscle fibers (Fig. 8). Muscle section was made from one animal per group, and a minimum of 150 fibers was measured per an animal.

4.6. Tissue Sampling and Extraction

Muscle samples for protein analysis taken from gastrocnemius and soleus were frozen by liquid nitrogen and were stored at -80°C. To make

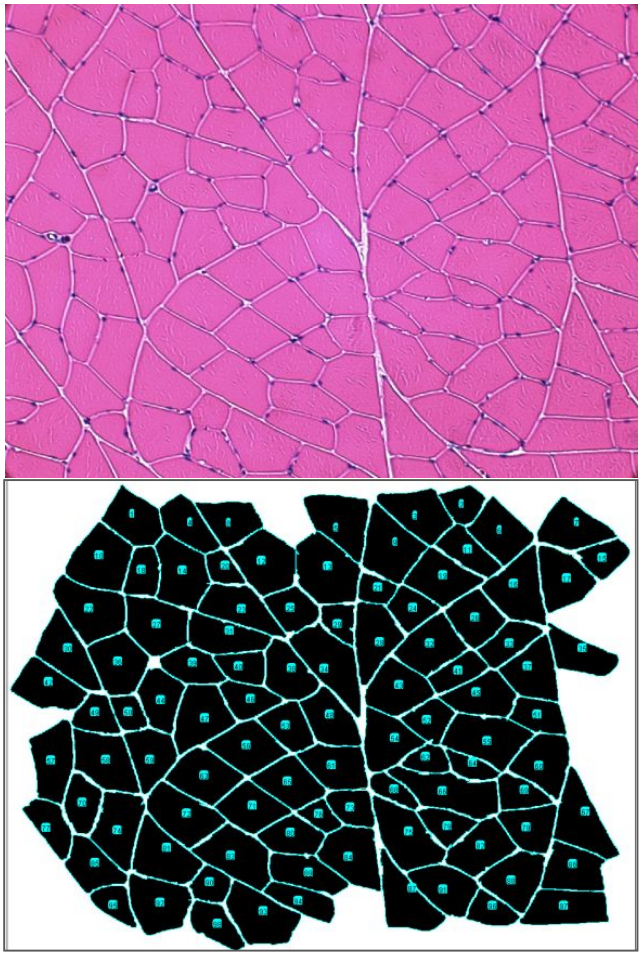

Figure 8. Cross-sectional area measured by analyses with ImageJ 1.44a software (Wayne Rasband, National Institutes of Health, USA, http://rsb.knfo.ih.gove/ij).

muscle homogenate, a section of 100 mg of muscle tissue was cut and homogenized with 200 µL of the lysis buffer containing EDTA, pepstatin A, leupeptin and aprotinin. The sonification and centrifugation were repeated three times and the supernatant was separated. The total protein contents were measured with an Absorbance Assay (280 nm) using Nanodrop, and the concentration was adjusted to 10 mg/mL with washing buffer. Subsequently, myofibrils were solubilized with 3 µL of SDS sample buffer. The SDS samples were heated at 50°C for 20 min and stored at 4°C before analysis.

4.7. MHC Isoform Composition

MHC isoform composition was analyzed using two samples, i.e., single muscle fibers (more than 20 per muscle) and protein homogenates. To prepare single muscle fibers, bundles of fibers were chemically skinned in relaxing solution containing 50% (v/v) glycerol at 4°C to disrupt membrane bound organells such as sarcolemma, t-tubules, SR, and mitochondria. After a 24 hour incubation at 4°C, the bundles were stored at -20°C (50, 51). Each single fiber was extracted under the stereomicroscope and dissolved with the lysis buffer (Fig. 9). The MHC composition was determined by 6% SDS-PAGE. The acrylamide concentration was 4% (v/v) in the stacking gel and 6% in the separating gel, and the gel matrix included 30% glycerol. SDS-PAGE was run at a constant voltage of 90 V for 30 min and 140 V for 5.5 hours (51). Protein homogenate mixtures of gastrocnemius and soleus were used as an MHC standard. Protein bands in the gels loaded with muscle homogenates were visualized with Coomassie Brilliant Blue. Densitometry was performed

Figure 9. Single muscle fiber extraction. Each single fiber was manually isolated from the muscle fiber bundles under a stereomicroscope. Arrow: a single muscle fiber.

using analytic software (Bio-1D Light, VilberLauret, French) to measure relative proportions of MHC isoforms. Gels loaded with single muscle fiber were subsequently stained with silver nitrite to identify MHC isoform of each single fiber and the relative proportion of the hybrid fibers in each muscle was determined.

5. Statistical Analysis.

The mean values for each group were compared using the nonparametric Mann–Whitney U test for two independent samples. Differences in the frequency of hybrid single muscle fibers among groups were tested by chi-square analysis. Analyses were performed using statistical software (SPSS version 18.0 for Windows; SPSS, Inc., Chicago, Illinois). A p value less than .05 indicated the presence of a statistical significance.

RESULTS

1. Foot print area

The foot print area was 3.23±0.14 cm^2 in the Ncon group. As shown in Fig. 16, a tendency toward decreasing foot print area was observed 4 weeks after denervation (p=.064). Foot print area showed significant improvement in the IES groups compared to that in the Dcon group (p=.034), which was not found in DES group. However, there was no significant difference of foot print area between DES and IES group (Fig. 10).

2. Extensor Postural Thrust Test

The percent motor deficits after 4 weeks of denervation injury in the Dcon, DES and IES groups were 36.8±15.6%, 28.8±17.5% and 36.7±7.4%, respectively, without any significant differences among groups (Fig. 11).

3. Ratio of CMAP amplitude

Fig. 12 shows the ratio of CMAP amplitude of the injured side to the intact side of the 4 groups. The ratio of CMAP was 0.16±0.18 in the Dcon group. Either the DES group or the IES group showed no significant difference in the ratio of CMAP amplitude compared to the Dcon group.

4. Muscle Wet Weight Comparison

Fig. 13 and 14 show the muscle wet weights of gastrocnemius and soleus at 4 weeks after the denervation injury. For the Dcon group, the muscle

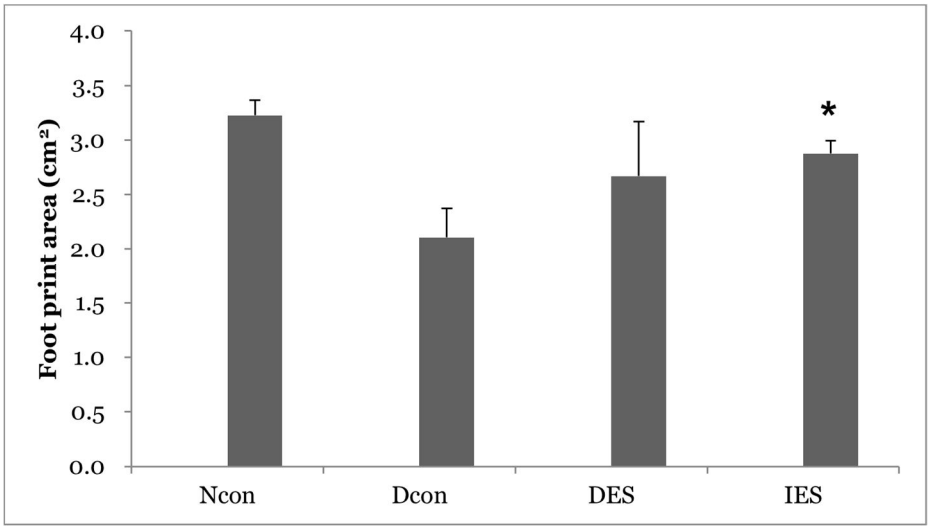

Figure 10. Foot print area of the right hind paw during stance using the CatWalk method at 4 weeks after the denervation injury. Ncon: group without any intervention; Dcon: group receiving no electromyostimulation after partial denervation; DES: group receiving delayed electromyostimulation; IES: group receiving immediate electromyostimulation. * $p<.05$ vs. Ncon.

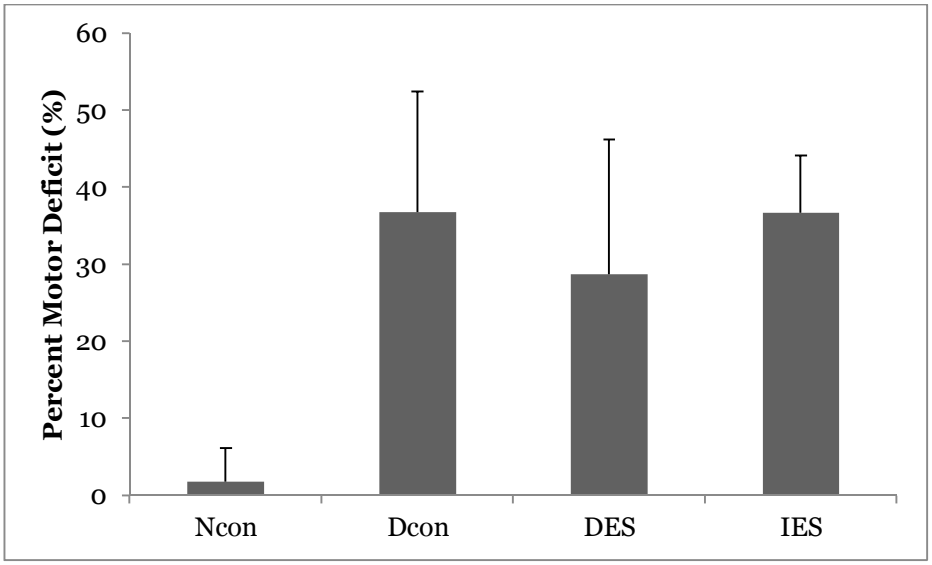

Figure 11. Percent motor deficit examined by extensor postural thrust test at 4 weeks after the denervation injury. Dcon: group receiving no electromyostimulation after partial denervation; DES: group receiving delayed electromyostimulation; IES: group receiving immediate electromyostimulation.

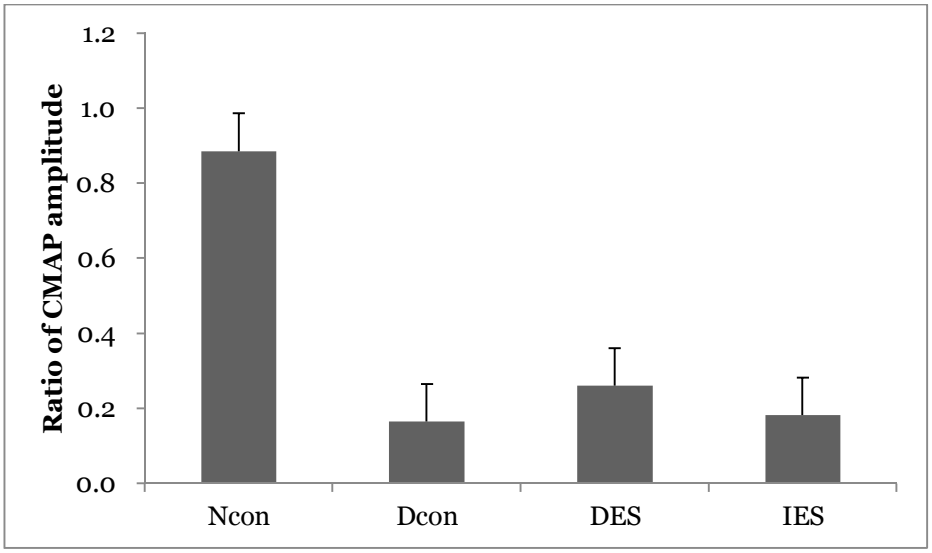

Figure 12. The ratio of the CMAP amplitude of the intact side to the injured side side at 4 weeks after the denervation injury. CMAP: compound muscle action potential; Dcon: group receiving no electromyostimulation after partial denervation; DES: group receiving delayed electromyostimulation; IES: group receiving immediate electromyostimulation.

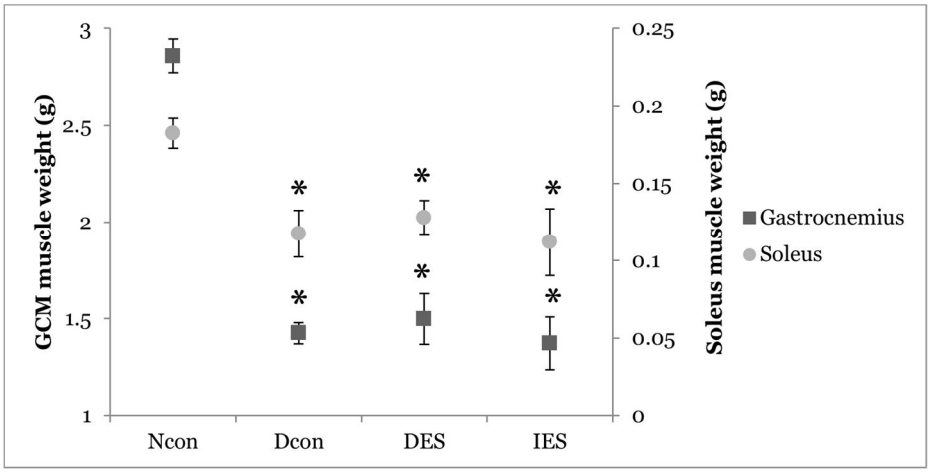

Figure 13. Muscle wet weights of the gastrocnemius and soleus at 4 weeks after the denervation injury. Ncon: group without any intervention; Dcon: group receiving no electromyostimulation after partial denervation; DES: group receiving delayed electromyostimulation; IES: group receiving immediate electromyostimulation. *, $p<.05$ vs. Ncon.

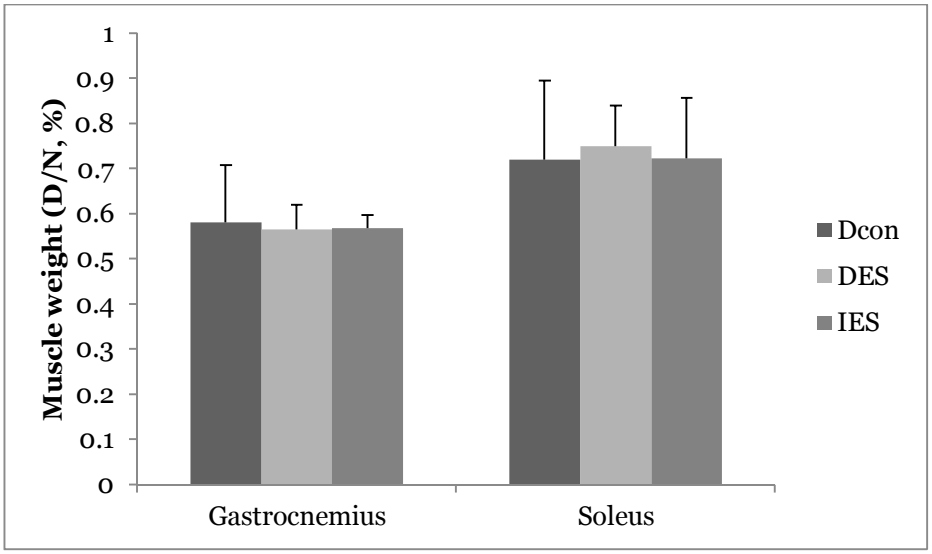

Figure 14. The ratio of denervated muscle wet weight to that of the intact side in gastrocnemius and soleus muscles at 4 weeks after the denervation injury. D/N: the ratio of denervated muscle wet weight to that of the intact side; Dcon: group receiving no electromyostimulation after partial denervation; DES: group receiving delayed electromyostimulation; IES: group receiving immediate electromyostimulation.

weights of gastrocnemius and soleus significantly decreased to 1.43±0.06 g (58.2±12.6% of the intact side, p=.021) and 0.12±0.02 g (72.1±17.4% of the intact side, p=.019), respectively. For Ncon group, the muscle wet weights of gastrocnemius and soleus were 2.86±0.09 g and 0.18±0.01 g.

The muscle wet weights of gastrocnemius and soleus in the DES group were 1.50±0.14g and 0.13±0.02g (56.6±6.0% and 74.9±9.0% of the intact side weights in gastrocnemius and soleus, respectively) which were not significantly different from those in the Dcon group after 4 weeks of the denervation. Similarly, the muscle wet weights of gastrocnemius and soleus in the IES group were 1.37±0.13 g and 0.11±0.01 g (56.8±3.0% and 72.3±13.3% that of the intact side, respectively), which were not significantly different from those in the Dcon group. There was no significant difference in the muscle wet weights of gastrocnemius and soleus between the DES and IES groups (Figs. 13, 14).

5. Morphometric Analysis

The representative images of muscle fiber cross-sections of the 4 groups of rats at 4 weeks after the denervation injury are illustrated in Fig. 15. The CSA of the gastrocnemius muscle fibers of each group are shown in Fig.16.

Compared to the Ncon group, significant decrease in the average muscle fiber CSA was observed in the Dcon group (2052.83±770.27 μm^2 vs. 1359.67±97.96 μm^2, p=.021). The DES group (1095.06±216.48 μm^2) also showed significantly smaller CSA compared to the Ncon group (p=.014).

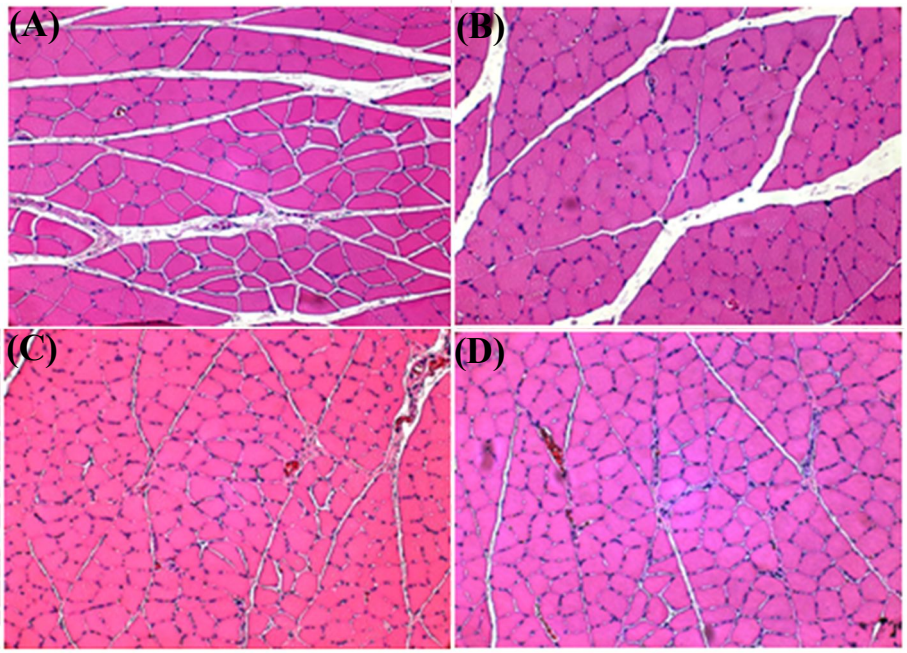

Figure 15. Representative images of muscle fiber cross-sections by H&E staining in the gastrocnemius muscles of (A) Ncon (the group without any intervention), (B) Dcon (the group receiving no electromyostimulation after partial denervation), (C) DES (the group receiving delayed electromyostimulation) and (D) IES (the group receiving immediate electromyostimulation) at 4 weeks after the denervation injury. Original magnification: 200x. H&E: Haematoxylin & Eosin.

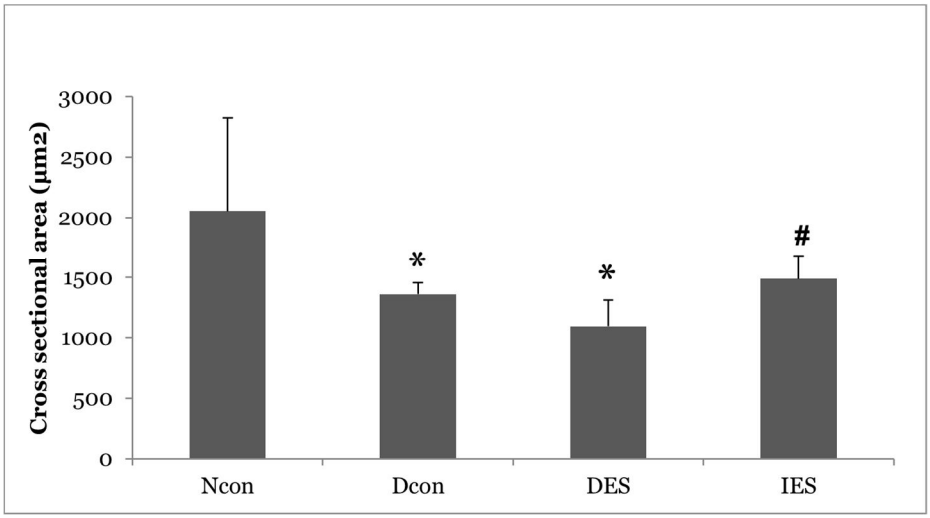

Figure 16. Cross-sectional area of gastrocnemius muscle fibers at 4 weeks after the denervation injury. Ncon: group without any intervention; Dcon: group receiving no electromyostimulation after partial denervation; DES: group receiving delayed electromyostimulation; IES: group receiving immediate electromyostimulation. *, $p<.05$ vs. Ncon; #, $p<.05$ vs. DES.

However, the CSA of the IES group was 1488.80±193.90 μm^2, which was similar to that of Ncon group and significantly greater than that of the DES group (p=.028).

6. MHC Isoform Analysis

The MHC of the Ncon gastrocnemius muscle homogenates contained 45.9±11.0% of type IIx, 41.5±27.2% of type IIb and 12.6±18.5% of type I isoform. Compared to the Ncon group, a significant increase in type IIx was observed in the Dcon and DES groups at 4 weeks after denervation (p=.021), which was not found in the IES group (Fig. 17A). There was no significant difference in the proportion of the MHC isoforms between Dcon and DES group. However, IES group showed significant decrease in type IIx (p=.014) and significant increase in type IIb (p=.027) compared to Dcon group. Type IIb of the IES group had a trend of increase compared to that of the DES group (p=.076).

The MHC of the Ncon soleus muscle homogenates contained 19.0±9.9% of type IIa isoform and 81.0±9.9% of type I isoform. At 4 weeks after denervation, the proportion of the MHC isoforms did not changed significantly in soleus muscles. The DES and IES groups also did not show significant changes in MHC proportion compared to control (Fig. 17B).

Based on the electrophoretic analysis for single muscle fibers, expression patterns of MHC isoforms were determined to be pure (expressing only one MHC isoform) or hybrid (expressing multiple types of MHC isoforms) such as type I/IIx, I/IIb, I/IIb/IIx, IIb/IIx in gastrocnemius or type

I/IIa in soleus. Fig. 18 shows the changes in the proportion of the hybrid MHC isoform after 4 weeks of the experiment. The proportion of hybrid single muscle fibers in the control gastrocnemius and soleus were 43.5±24.6% and 11.2±9.9%, respectively. Four weeks after the denervation, the hybrid proportion in gastrocnemius was significantly increased (p=.000). In the IES group, the proportion of hybrid fibers in gastrocnemius significantly decreased compared to that in the Dcon group (p=.000), which was not found in the DES group. At 4 weeks after denervation, the hybrid proportion did not change significantly in soleus. The DES group showed a significant increase in hybrid proportion compared to those of the other three groups (p<.05), which was not found in the IES group (Fig. 18).

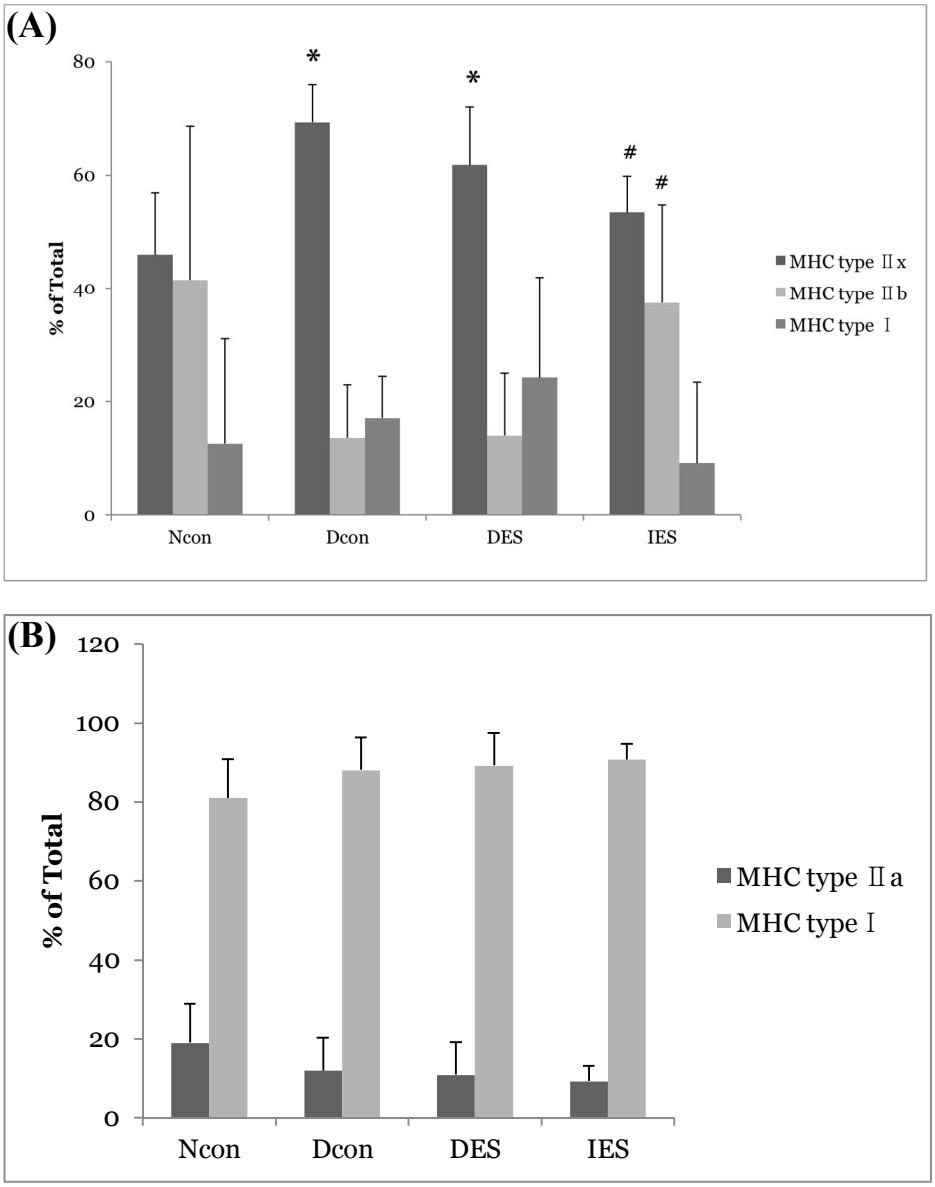

Figure 17. The proportion of the MHC isoforms in gastrocnemius (A) and soleus (B) at 4 weeks after the denervation injury. Ncon: group without any intervention; Dcon: group receiving no electromyostimulation after partial denervation; DES: group receiving delayed electromyostimulation; IES: group receiving immediate electromyostimulation. *, $p<.05$ vs. Ncon; #, $p<.05$ vs. Dcon.

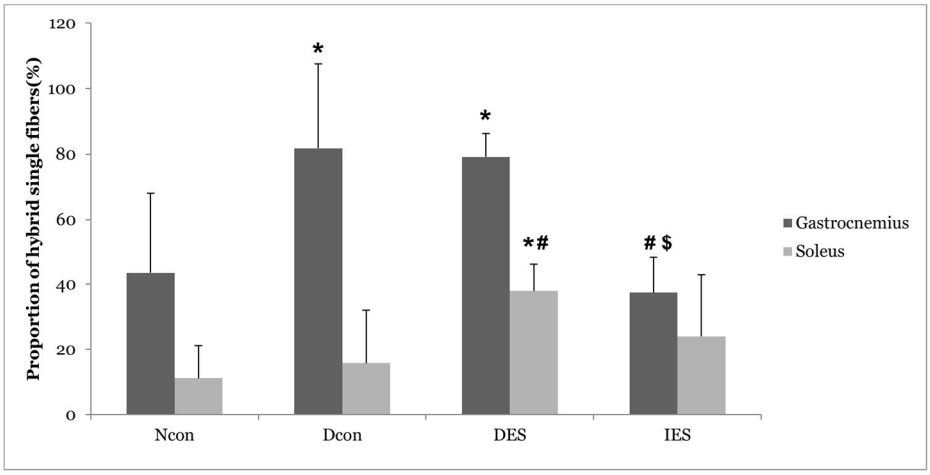

Figure 18. The changes of the hybrid MHC isoform proportion in analysis of single muscle fibers at 4 weeks after the denervation injury. Ncon: group without any intervention; Dcon: group receiving no electromyostimulation after partial denervation; DES: group receiving delayed electromyostimulation; IES: group receiving immediate electromyostimulation. *, $p<.05$ vs. Ncon; #, $p<.05$ vs. Dcon; $, $p<.05$ vs. DES.

DISCUSSION

Electromyostimulation (EMS) is a direct muscle stimulation which causes muscle contraction. Although EMS has been successfully applied for the treatment of denervated muscle, only limited information is available about the exact effects of EMS particularly on the MHC isoforms or the optimal timing for the application of EMS. Therefore, this study examined the effects of EMS on the denervated muscles including potential alterations in the MHC isoforms, depending on the application timing.

When the effects of the immediate vs. delayed initiation for the EMS were compared, the immediate application of EMS showed the potential to reverse some of the changes induced by denervation. The analysis of MHC isoform proportions revealed that the expression of MHC type IIx in gastrocnemius was increased in 4 weeks after denervation. However, immediate EMS was found to be able to return this increase in MHC type IIx proportion comparable to the control level, whereas delayed EMS was not. The expression ratios of each MHC type in soleus muscle was not affected either by denervation or EMS.

The changes induced by denervation in gastrocnemius muscle were consistent with the results in fast-twitch muscle in previous reports (15). EMS of a tongue muscle induced fast-to-slow shift in the MHC content in normal rabbit (52). Application of EMS to the tibialis anterior muscle also led to fast-to-slow shift in the MHC content in normal rats (53). To date, however, there has been no reports on the EMS-induced alterations in the MHC expression in

partial denervation. In this study, when applied immediately after denervation, EMS led to the changes in the MHC isoform proportion in gastrocnemius, which was opposite to denervation-induced changes. These result suggests that the immediate EMS may be a good candidate as a treatment for the denervation-induced muscle atrophy based on its reversing effect of denervation-induced consequence.

However, the effects of the immediate EMS was not observed in slow twitch type I fiber soleus muscle. The follow-up period of 4 weeks after the denervation injury in the present study might not be sufficient to clarify the plastic effect of EMS in partially denervated muscle. It has been well described in the literature that slow-twitch muscles exert antigravity function (54) and are more responsive to immobilization or disuse compared to fast-twitch muscles. Further studies on the optimal treatment for the recovery of muscle atrophy should be designed considering the cause of muscle atrophy (denervation or disuse) and muscle fiber types (fast or slow-twitch).

The MHC isoform analysis of the single muscle fiber also suggested positive effects of the immediate EMS. Denervation increased the proportion of hybrid fiber (muscle fibers co-expressing multiple MHC isoforms) after 4 weeks in gastrocnemius. The increased proportion of the hybrid fibers were not found in the group of rats which received EMS immediately after denervation. The fraction of the hybrid fibers for the IES group was similar to that for the control group in gastrocnemius, while delayed EMS had no effect on a predominance of hybrid muscle fibers in the denervated gastrocnemius muscle. In soleus muscle, neither denervation nor immediate EMS appeared

to change the proportion of hybrid fibers. However, higher proportion of hybrid single fibers in soleus were observed in DES treated group.

Hybrid fibers have potential to transform into one pure fibers type after reinnervation. It is now widely accepted that the fraction of MHC hybrids is increased in muscles in molecular and functional transformations (11, 55-57). Thus, the decreased hybrid fibers proportion in the IES group for the fast twitch gastrocnemius muscle may imply that immediate EMS can rapidly stabilizes denervation-induced changes, which cannot be achieved by delayed EMS. However, those finding were not evident in slow twitch soleus muscle. Denervation or immediate EMS did not show any apparent changes in the hybrid proportion. Delayed EMS may accelerate the formation of hybrid fibers in soleus muscle.

The results that the immediate EMS reversed MHC proportion close to normal proportion and decreased hybrid proportion of single fibers in denervated gastrocnemius indicate that immediate EMS had an effect to facilitate reinnervation-induced changes in MHC expression. Nevertheless, it should be noted that this effect of immediate EMS may not directly lead to the improvement of the overall muscle function. Although CSAs of muscle fibers increased to similar to the control level only in the immediate EMS treated rats, immediate EMS in denervated muscle did not make significant changes in ratio of CMAP amplitude, percent motor deficit or muscle wet weight. Only foot print areas of the denervated limb in the immediate EMS group showed significant increase compared to that of denervation control group. A longer follow-up may be needed to evaluate whether immediate EMS can

recover the muscle mass or motor function as well as denervated changes in contractile protein.

A recent study showed harmful effects of EMS based on six EMS sessions on the denervated tibialis anterior muscle applied every other day starting from day 3 post-injury until the end of the experiment (day 14) in the initial phase after sciatic nerve crush injury in rats (39). In the study, the EMS was associated with a delay of functional recovery, muscle hypoexcitability and severe muscle atrophy (39). Other investigators, on the contrary, reported that chronic EMS partially favors the recovery of mechano- and metabosensitivity in a denervated muscle after nerve repair by self-anastomosis (58). These contradictory results about the effects of EMS seem to be related to the different types of electrode (using implanted vs. surface electrodes), onset time to stimulation, the amount of stimulation (42), the interval between each stimulation (41), and the types of lesion (transection vs. crush injury).

Our data indicate that the EMS may be able to inhibit denervation-induced muscle atrophy when EMS is administered very early after the nerve injury. It should be noted that immediate EMS was applied after 1 day from the injury, before the recovery by reinnervation began in the denervation model. Following muscle denervation, embryonic MHC, which is not expressed in adult healthy muscles, was expressed in some denervated fibers as well as in small activated satellite cells and the maximal expression was observed 2 to 3 weeks after denervation (59). To reverse or inhibit these degenerative changes in denervated muscle, application of EMS earlier may

be more effective. Our data suggest that starting EMS immediately after denervation injury may be more helpful in preventing denervation-induced atrophy than starting 2 weeks after the injury.

The follow-up period of 4 weeks after the injury may be long enough considering life cycle of rats. However, a further study with longer follow-up period would be needed to confirm the changes observed in the current study as well as to determine the moment of total functional recovery. In our previous study, it has been observed that the degenerative changes predominated for 4 weeks after injury, and most of the regeneration occurred actively from 4 to 8 weeks (36). Therefore, 4 weeks follow-up period does not seem to be sufficient to determine the effects of EMS on the recovery of muscle mass and motor function in the present study protocol. In addition, in order to induce sufficient muscle contraction, our study used 1-Hz frequency and 100-ms stimulation duration. The motor unit activation caused by this stimulation might not be as physiologic as voluntary activation. Future studies with an EMS method which can induce more physiologic muscle activity will be required.

CONCLUSION

The EMS applied immediately after the nerve injury increased the CSA of the denervated muscle fibers, reversed denervation-related changes of MHC isoform expressions and reduced hybrid single muscle fibers in partial denervation, whereas delayed EMS had no effect. These results indicate that the immediate EMS is more effective in recovery of denervation induced MHC changes compared to the delayed EMS.

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국문 초록

서론: 탈신경 손상에 의한 근위축의 치료로 전기자극을 근육에 적용하는 것은 임상적으로 널리 이용되어 왔다. 그러나, 근육내전기자극의 탈신경 손상에 의한 근위축에 대한 효과나, 적절한 시작 시기에 대해서는 논란이 계속되고 있다.

목표: 본 연구의 목적은 근육내전기자극이 마이오신 중쇄(myosin heavy chain)와 관련하여 탈신경된 근육에 미치는 효과를 알아보고, 탈신경 손상 직후와 손상 2 주후에 각각 근육내전기자극을 시작하는 것을 비교하여 적절한 시작시기를 결정하고자 한다.

방법: 8 주령의 Sprague-Dawley 흰쥐 18 마리 중 14 마리를 대상으로 우측 좌골 신경에 압궤손상을 가하여 부분 탈신경 모델을 만들었다. 부분 탈신경된 14 마리 중 5 마리씩을 즉시 자극군과 지연 자극군으로 나누어 각각 손상 1 일 후부터 14 일까지, 손상 15 일 후부터 28 일까지 근육내전기자극을 2 주간 시행하였다. 남은 4 마리는 근육내전기자극을 가하지 않는 탈신경 대조군으로 사용하였다. 탈신경 손상을 주지 않은 4 마리는 정상 대조군으로 하였다. 탈신경 손상 4 주 후, 전기생리학적 평가와 행동 평가를 시행하고, 모두 희생하여 비복근과 가자미근을 적출한 후, 근육

습중량 및 근섬유 단면적을 측정하고, 단백질 균질액과 단일근섬유에서 마이오신 중쇄 이소형 분석을 시행하였다.

결과: 즉시 자극군에서, 탈신경된 비복근의 근섬유 단면적이 유의하게 증가하였고, 탈신경에 의한 마이오신 중쇄 이소형 비율의 변화가 감소하였으며, 하이브리드 단일근섬유의 비율 증가가 완화되었다. 그러나, 지연 자극군에서는 유의한 효과가 나타나지 않았다.

결론: 본 연구의 결과는 탈신경 손상 직후에 근육내전기자극을 시행하는 것이 손상 2 주 후부터 시행하는 것보다 탈신경 손상에 의한 마이오신 중쇄의 변화를 회복시키는 데 좀더 효과적임을 나타낸다.

주요어: 근육내전기자극, 근위축, 탈신경, 마이오신 중쇄

학 번: 2010-30561