Training at non-damaging intensities facilitates recovery from muscle atrophy

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―マウス萎縮筋に対する等尺性筋力トレーニングによる検証―）

名古屋大学大学院医学系研究科
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平成２８年度学位申請論文

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CONTENTS

Abstract

Introduction: Resistance training promotes recovery from muscle atrophy, but optimum training programs have not been established. We aimed to determine the optimum training intensity for muscle atrophy. **Method:** Mice recovering from atrophied muscles following 2 weeks of tail suspension underwent repeated isometric training with varying joint torques 50 times per day. **Results:** Muscle recovery assessed by maximal isometric contraction and myofiber CSAs were facilitated at 40% and 60% maximum contraction strength (MC), but at not at 10% and 90% MC. At 60% and 90% MC, damaged and contained smaller diameter fibers were observed. Activation of myogenic satellite cells and a marked increase in myonuclei were observed at 40%, 60%, and 90% MC. **Discussion:** The increases in myofiber CSAs were likely caused by increased myonuclei formed through fusion of resistance-induced myofibers with myogenic satellite cells. These data indicate that resistance training without muscle damage facilitates efficient recovery from atrophy.

Keywords: resistance training; muscle atrophy; isometric contraction; intensity; recovery; torque

1. Introduction

1-1. Plastisity of skeletal muscle

Skeletal muscle is a highly plastic tissue that exhibits hypertrophy and atrophy in response to increased and decreased mechanical loading, respectively. For instance, unloading using tail suspension results in soleus muscle atrophy, and mass and size of the muscle decrease by approximately half within 2 weeks in rodents. These atrophied muscles recover from their normal size after another 2 weeks of reloading[1-3].

Skeletal muscle is mostly composed of myofibers. Therefore, the increase in the thickness of the myofibers, or the number of myofibers are deemed necessary to increase in size of the skeletal muscle. On the other hand, the decrease of the thickness of myofiber, or the number of myofibers are considered to be necessary to atrophy of skeletal muscle belly thickness. In practice, the thickness of myofibers and the number of myofibers increases during the skeletal muscle hypertrophy induced by the resistance training[4-6]. In contrast, the thickness and the number of myofibers decrease during the muscle atrophy induced by unloading[7-9]. However, the recent study suggested that the change in the muscle belly thickness was not associated with chenages in the actual numbers of myofibers[10]. Therefore, I considered that the changes in the thickness of myofiber, not number of myofibers play a major role in the plasticity of skeletal muscle tissue.

When the thickness of myofibers increase, it is necessary to promote the proteins synthesis and/or to

inhibition the proteolysis. Actually, it is well established that the Akt/mTOR/p70S6K/4E-BP1 signal cascade that protein synthetic signaling are enhanced during hypertrophy of myofibers due to the exercise or mechanical stimulation[11-13]. Similarly when the thickness of myofibers decrease, it is necessary to inhibition the proteins synthesis and/or to promote the proteolysis. It has also been reported that the protein degradation signals are activated in the atrophied myofibers due to disuse[1].

The proteins that form myofibers are encoded by the DNA of myonuclei, which suggests a strong relationship between the number of myonuclei and the size of myofibers [14]. For example, exercise significantly increases the number of nuclei during hypertrophy of normal muscle [15,20]. Moreover, muscle atrophy caused by unloading reportedly involves a reduction in the number of myonuclei by apoptosis along with a decrease in the size of myofibers [7-9,16,17]. In general, myonuclei are believed not to multiply after their terminal differentiation in mammals[18]. In addition, there was no increase in the number of myonuclei when myogenic satellite cells were inactivated by irradiation[19]. These results suggest that satellite cells proliferate and differentiate into myoblasts, which then fuse with exiting myotubes when increasing the thickness of myofibers during muscle hypertrophy[19,3]. However, the cellular and molecular processes have not been elucidated in detail.

1-2. Effect of resistance training for muscle hypertrophy

Especially in animal experiments, muscle strength has been often used as indicator to measure the

function of skeletal muscle. In the factors defining the muscle strength, although there are neurological factors such as an increased firing frequency[20,21] and synchronization of motor units[22,23], it also have been known that the proportional relationship between myofiber size (cross-sectional area) and muscle strength[24-29]. Thus, functional changes due to the above-mentioned change of the thickness of myofibers are appeared to muscle strength.

For these reason, disuse muscle atrophy reduced muscle strength. Muscle strength weakness caused by muscle atrophy is a factor that limitations of activities of daily living, result to limit the early recovery from the original disease. Therefore, it is important that facilitating of recovery from disuse muscle atrophy for rehabilitation after forced rest or inactivity. In order to realize facilitating of recovery from muscle atrophy, the resistance training is practiced in physical therapy. Some studies have proposed appropriate resistance loads for the purpose of strengthening normal muscles [30,31] , and resistance training at an intensity that can be repeated 8–12 times [60%–80% one-repetition maximum (1RM)] is effective for developing muscle hypertrophy in healthy subjects [30]. In addition, animal studies have supported these training effects [25-27]. However, there is no established training program for patients with muscle atrophy.

1-3. Optimal intensity of resistance training for atrophied muscle

Animal studies of the training effects in unloading-induced atrophied rats using wheel or treadmill

running [32,33] did not establish an effective training intensity or an effective training period. Therefore, rehabilitative training to recover the strength of atrophied muscles is often performed according to the recommendations for normal muscle. It is unclear whether resistance training is effective for the management of muscle atrophy. We had previously investigated whether resistance training with stand-up exercise using operant conditioning was effective in facilitating the recovery of mice from muscle atrophy induced by tail suspension (TS) [34]. Our results revealed that stand-up exercise training facilitated recovery of the thickness of myofiber in atrophied muscles. Myofiber cross-sectional areas (CSAs) atrophy to 50% of their normal thickness in just 2 weeks of TS [35-40,34], and reloading recovers the level of normal muscle in 2–4 weeks [1,3,2] (Fig. 1A). On the other hand, 8–16 weeks are required to cause muscle hypertrophy in normal muscle [25,26,24,28,29]. Thus, inducing recovery of atrophied muscle to normal thickness is faster than that of normal muscles, suggesting that different mechanisms may be involved in inducing recovery of atrophied muscle and hypertrophy of normal muscles.

We previously reported that the number of myonuclei per myofiber in atrophied muscle increases more than that of the control muscle within 4 days after stand-up exercise training [34] (Fig. 1B). This increase was not observed in the muscles of mice that did not undergo similar training during the reloading period. Thus, an increase in the number of myonuclei would therefore be a pivotal component of the mechanism of recovery from muscle atrophy. However, the relationship between changes in the number of myonuclei and intensity of resistance training for recovery from muscle

atrophy is unknown.

1-4. Objective of this study

The aim of this study was to clarify this relationship between the intensity of training and the facilitating effect of recovery from atrophied muscle, we prepared a device given the exercise to mice with reproducible strength, and electrophysiologically or histologicaly analyzed the change of the muscle strength, myofiber size, number of myonuclei and the occurrence of muscle injury.

2. Methods

2-1. Animals

All experiments were approved by the Animal Care Committees of Nagoya University (No. 025-023) and Nagoya Gakuin University (No. 2007-007). Eleven-week-old male Institute of Cancer Research (ICR) mice (Japan SLC, Shizuoka, Japan) were housed at room temperature (25°C) under a 12-h light–dark cycle and were provided food and water *ad libitum*. To induce hind-limb muscle atrophy, all mice were suspended by the tail for 2 weeks so that only the forelimbs touched the bottom of the cage [39,41]. Briefly, mice were anesthetized using isoflurane inhalation (1.0%; Abbott, Tokyo, Japan), and the tail was attached to a suspension harness. The forelimbs of the mice reached the ground during TS; therefore, they were free to move around the cage and had access to food and water. To

investigate the effects of the intensity of resistance training on recovery from muscle atrophy, we divided the atrophied mice into 4 groups that were subjected to isometric training using different intensities described below. We included 2 control groups (n/group = 6, Fig. 1A). One group was fed normally but was not trained after TS (NT group), and the other was not subjected to TS or resistance training (CON group).

2-2. Quantitative muscle contraction using electrical stimulation and analysis of maximum isometric torque

To investigate the effects of resistance training intensities on recovery from muscle atrophy, mice were subjected to 50 repetitions of isometric contraction exercise, and electrical stimulation was applied to the lower limbs every day for 7 days (on DAY0-6) during the reloading phase after TS (Fig. 2A). To achieve quantitative isometric contraction using electrical stimulation, we developed a system comprising an electric stimulator (SEN-3301, Nihon Kohden, Tokyo, Japan) and a pressure sensor (Fig. 2B). Mice were anesthetized using isoflurane inhalation, and electrical stimulation was applied to the posterior surface of the skin of the lower limbs. To attach a surface electrode (custom-made, Bio Research Centre, Nagoya, Japan), the skin of the hindlimbs was shaved and cleaned with ethanol. Viscous electrical conductive gel (CR-S, Sekisui Plastics, Osaka, Japan) was applied between the electrodes and the skin. The electrodes were fixed with adhesive tape to the surface of the

myotendinous junction and a 6 mm proximal locus. Isometric plantar flexion torque (*T*) was calculated from the pressure applied to a footplate (*F*) and the distance from the axis of ankle joint to the sensor (*r*) as follows:

$$T = Fr$$

To assess the effects of isometric contraction intensities on recovery of muscle strength from muscle atrophy, maximal plantar flexion isometric torque was measured 4 hours before isometric exercise of anesthetized mice each day after completion of TS (on DAY 0-6, Fig. 1A). With the same device used for isometric exercise, plantar flexor muscles were percutaneously stimulated via surface electrodes, and maximal plantar flexion was evoked using a supramaximal tetanic current (100 Hz frequency, 1.0 ms duration, 650 ms train duration, and 5.0 mA current). In preliminary experiments, we gave electrical stimulus frequencies of 25, 50, 75, 100, and 125 Hz. The maximal torque was observed at 100 Hz (Fig. 3). We also checked visually that ankle dorsiflexion was not induced by the electrical stimulation. As a result, we decided to use an electric stimulus of 5 V at 100Hz for functional analysis of maximum isometric torque. Similar results have been reported elsewhere[42,43].

For isometric contraction exercise, electrical stimulation parameters (5V, 40 Hz frequency, 2.0 ms duration, 250 ms train duration) were used, and the torque was adjusted by changing the value of the electrical current. The intensities of isometric training were set to 10%, 40%, 60%, and 90% of maximum isometric plantar flexion torque (described above), which were measured immediately after

the completion of TS [10%, 40%, 60%, and 90% maximum contraction (MC) groups]. The torques for 10%, 40%, 60%, and 90% of the maximum isometric torque were ~1.0, 3.0, 5.0, and 8.0 mNm, respectively. The current value used in the 10%, 40%, 60%, and 90% MC groups were adjusted based on the exerted torques at the onset of stimulation, at 1.2–1.6 mA, 2.0–2.4 mA, 2.8–3.2 mA, and 4.4–4.8 mA, respectively. An ~10% decrease in torque was observed after 100, 50, 30, or 2 repetitions of isometric contractions in the 10%, 40%, 60%, and 90% MC groups, respectively (Fig. 2C–F). From the calculation of mouse body weight, and the length of hindlimb and foot, the intensity of 40% MC is equivalent to that for the stand-up exercise which facilitates the recovery from muscle atrophy [34].

2-3. Immunohistochemical and histological assays

To assess the effects of the isometric contraction intensities on recovery from muscle atrophy with histological methods, the soleus muscles were collected from the mice under sodium pentobarbital anesthesia (0.05 mg/BWg IP, Somnopentyl, Kyoritsu Seiyaku, Tokyo, Japan) on DAY3 and DAY7 of training (Fig. 2A). After weighing, the muscle was then frozen in isopentane precooled in liquid nitrogen. Subsequently, 8-μm-thick frozen transverse sections were prepared through the widest area of the muscle (between 3 mm and 5 mm from the distal tendon). Frozen transverse sections were stained with hematoxylin-eosin (H-E) and photographed under a microscope using a digital camera (Ds-Ri1, Nikon, Tokyo, Japan). Images were analyzed using ImageJ software to measure myofiber

cross-sectional area (CSA).

H-E sections were analyzed to detect muscle damage. Next, to detect nascent myofibers, the sections were incubated along with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, MO, USA), an anti-laminin antibody, and an anti-developmental myosin heavy chain (dMHC) antibody (see details below) so that nuclei, basement membranes, and newly generated myofibers could be visualized, respectively. The sections were fixed with 4% paraformaldehyde for 10 min, washed in phosphate-buffered saline (PBS), and blocked with 3% bovine serum albumin in PBS overnight. After washing in PBS, sections were incubated for 60 min at 37°C with mouse anti-dMHC monoclonal antibody (1:40, VP-M664, Vector, CA, USA) and a rabbit anti-laminin polyclonal antibody (1:1000, L9393, Sigma-Aldrich). After washing, immunolabelled sections were incubated in the dark for 45 min at 37°C with Alexa Fluor 568-conjugated goat anti-rabbit IgG antibody (1:400, A11008, Molecular Probes, CA, USA), Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (1:400, A11029; Molecular Probes), and DAPI (10 μg/mL).

To determine the number of myonuclei, the sections were double-stained with DAPI and an anti-dystrophin antibody (see details below) to visualize the sarcolemmal membrane. After fixing and blocking as described above, sections were incubated for 60 min at 37°C with a rabbit anti-dystrophin polyclonal antibody (1:400, sc-15376, Santa Cruz, TX, USA). After washing, immunolabelled sections were incubated in the dark for 45 min at 37°C with Alexa Fluor 568-conjugated goat anti-

rabbit IgG antibody (1:400, A11008, Molecular Probes) and DAPI. Sections were photographed using a fluorescence microscope (TE300, Nikon, Tokyo, Japan) and images were analyzed using ImageJ. The numbers of DAPI-stained nuclei within the anti-dystrophin-stained sarcolemma were counted to estimate the mean number of myonuclei per myofiber. As a sorting criterion for myonuclei, we considered that the nuclei that rim the inside of the dystrophin ring were myonuclei, excluding those that overlapped the ring. We excluded nuclei that we were unable to judge. We considered that the myonuclei that did not contact the dystrophin ring were central nuclei, and we counted myofibers with central nuclei in the whole muscles. The proportion of the number of myofibers with central nuclei to that of total myofibers counted was noted by percentage.

To determine whether myogenic satellite cells were in an activated or differentiated stage, sections prepared as described above were triple-immuno-stained with a mouse anti-Pax7 antibody (1:250, Developmental Studies Hybridoma Bank, IA, USA), a rabbit anti-MyoD antibody (1:200, sc-760, Santa Cruz), and DAPI. Immunolabelling was visualized using an Alexa Fluor 488-conjugated goat anti-mouse IgG (1:400, A11029; Molecular Probes) or Alexa Fluor 568-conjugated goat anti-rabbit IgG (1:400, A11036; Molecular Probes). Other sections were incubated with a goat anti-MyoD antibody (1:200, sc-31942; Santa Cruz), a rabbit anti-Myogenin antibody (1:200, sc-576; Santa Cruz), and DAPI. Immunolabelling was visualized using Alexa Fluor 568-conjugated donkey anti-goat IgG (1:400, A11057; Molecular Probes) or an Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:400,

A11008; Molecular Probes).

2-4. Statistics

For multigroup comparisons, we used a repeated measures ANOVA followed by pairwise comparisons using the Bonferroni correction. A *P*-value of <0.05 was defined as statistically significant.

3. Results

3-1. Recovery of maximal isometric torque depends on the intensity of resistance training

Changes in maximal isometric torque during resistance training were determined following TS (Fig. 4). Immediately after completing TS (on DAY0), the maximal isometric torque was 58% lower than that of the CON group. One day after training (on DAY1), the maximal isometric torque in the 40% and 60% MC groups were significantly greater than that of the NT group. Furthermore, the differences between the CON and the 40% or 60% MC groups were not significant 3 days after isometric contraction exercise (on DAY3). There were no significant differences between the NT and the 10% or 90% MC group during the 6 days of isometric contraction exercise following TS (on DAY6).

3-2. Recovery of myofiber CSA depends on the intensity of isometric contraction exercise

Seven days after isometric contraction exercise (on DAY7), the muscle wet weights of the NT and

10% MC groups were lower than that of the CON group (Table 1). The muscle wet weights in the 40% and 90% MC groups were heavier than those in the NT group and did not differ from the CON group. The muscle wet weight of the 60% MC group did not differ from those in the CON and NT groups 7 days after isometric contraction exercise (on DAY7). The mean myofiber CSAs of all exercise groups were significantly smaller than that of the CON group 3 days after isometric contraction exercise (on DAY3, Fig. 5A). However, the median values of CSA in the trained groups were larger than those of the NT group. Among the trained groups, the value of the seventy-fifth percentile of the 40% MC group was larger than those of the 10%, 60%, and 90% MC groups. The median value of the 60% MC group was greater than those of the 10%, 40%, and 90% MC groups. Large CSA myofibers (>1500 μm^2) were observed in the 40% MC, 60% MC, and CON groups (Fig. 5B). After 7 days of isometric contraction exercise following TS (on DAY7), the myofiber CSA of the 40% MC group was significantly larger than that of the NT group and not significantly different from that of the CON group (Fig. 5C). The myofiber CSA of the 60% group was significantly larger than that of the NT group but significantly smaller than that of the CON group. Although the muscle weight of the 60% MC group was not significantly different from the NT or CON groups as described above, the muscle weight of 60% MC group tended to be heavier than that of NT group but tended to be lighter than that of CON group. These results are partially consistent with the CSA data. No significant difference of myofiber CSA was observed between the 10% MC and NT groups. The values of the seventy-fifth

percentile of the 40% and 60% MC groups were not significantly different from those of the CON group. The values of the twenty-fifth percentile of the exercise groups were smaller than those of the CON group. The distributions of myofiber CSA histograms of the 40% and 60% MC groups were similar to those of the CON group (Fig. 5D). The distributions of the 10% and 90% MC groups were not significantly different from those of the NT group. Small CSA myofibers, particularly those ranging from 100–500 μm^2, were observed only in the 60% and 90% MC groups.

3-3. Isometric contraction exercise at excessive intensity damages myofibers

The presence of muscle damage at 3 or 7 days after resistance training following TS was assessed using H–E staining (Fig. 6A, C). Three days after isometric contraction exercise (DAY3), myofibers infiltrated with mononuclear cells were observed in the muscles of the 60% and 90% MC groups. However, those fibers were observed at a low frequency in the muscles of the 10% and 40% MC groups. The dMHC-positive myofibers were observed notably in the muscles of the 60% and 90% MC groups 3 days after isometric contraction exercise (DAY3, Fig. 6B). Seven days after isometric contraction exercise (DAY7), myofibers with infiltrating mononuclear cells (Fig. 6C) or those that expressed dMHC (Fig. 6D) were not observed in any of the exercise groups. Staining of myofiber membranes and nuclei revealed that the population of myofibers with a central nucleus was significantly greater in the 90% MC group than in the NT and CON groups 7 days after isometric

contraction exercise (DAY7, Fig. 6D, E). Myofibers with a central nucleus were infrequent in all groups 3 days after isometric contraction exercise (DAY3, Fig. 6B).

3-4. Isometric contraction exercise increases the number of myonuclei during recovery from muscle atrophy

The numbers of myonuclei were determined with images of myofibers obtained from the muscles 7 days after isometric contraction exercise following TS (DAY7) such as Fig. 6D, F. The numbers of myonuclei were significantly higher in the 40%, 60%, and 90% MC groups than in the CON group. The highest number of myonuclei was observed in the 40% MC group. No significant difference in the numbers of myonuclei was detected between the 10% MC group and the NT or CON groups. The nuclei were co-stained with anti-Pax7 and anti-MyoD, and MyoD and myogenin were detected in the 40%, 60%, and 90% MC groups (Fig. 7A-C).

4. Discussion

4-1. Effects of isometric contraction exercise intensities on the recovery from muscle atrophy

Different intensities of resistance training lead to varying adaptations in normal muscles [30,31,44-46]. For example, resistance training at an intensity that can be repeated 1–2 times (90% 1-repetition maximum; 1RM) improves muscle power, an intensity repeated 8–12 times (60%–80% 1RM) causes

muscle hypertrophy, and an intensity repeated 30–40 times (40%–50% 1RM) improves muscle endurance [31]. For example, during resistance exercise at 90% 1RM, the amount of discharge per motor unit increases [44-46] . During exercise at 60%–80% 1RM, fast-twitch fibers that are prone to hypertrophy [47] are frequently recruited [31,44,45] . At 40%–50% 1RM, the number of blood vessels [48] and mitochondria [49] increases, resulting in improved metabolic capacity of muscle. Therefore, different intensities of resistance training exert differential effects on the recovery from muscle atrophy.

Atrophied muscle is more fragile than normal muscle [50,51]. The exercise periods required for development of hypertrophy in normal muscle [28,29] and for recovery of the thickness of atrophied muscles [34] differ, suggesting that the exercise intensity for recovery of atrophied muscle should be different from that for hypertrophy of normal muscle. Although Adams et al[52] have suggested that combined isometric, concentric, and eccentric resistance exercise during TS prevents muscle atrophy in rats, and thus the protocol might inhibit the atrophy process, the adequate intensity of the resistance exercise for atrophied muscle after TS has not been defined. In our study, we assessed the effects of isometric contraction exercise at different intensities on the recovery from muscle atrophy induced by TS. The results demonstrate that isometric contraction exercise performed at 40% MC (~3 mNm) promotes the fastest recovery of torque of maximum isometric muscle contraction associated with marked recovery of myofiber CSA.

Isometric contraction exercise at 10% MC (~1 mNm) did not facilitate recovery. The torque loaded

on the ankle joint while walking on a flat surface is ~1 mNm in mice reared normally [53]. In contrast, the torque loaded on the ankle joint when mice stand on their tiptoes is approximately 3 mNm when weight and foot length are taken into account. We previously reported that exercise training with mice standing on their tiptoes achieved a greater extent of recovery from muscle atrophy compared with mice reared normally [34]. This finding is in agreement with the results reported here. Thus, our method may prove useful for evaluation of the effects of resistance training intensities on the recovery from muscle atrophy.

We found that isometric contraction exercise at 60% MC increased the level of recovery of the maximum isometric plantar flexion torque equivalent to that of the 40% MC group 3 days after the isometric contraction exercise following TS. However, the effect on the myofiber CSAs of the 60% MC group was less pronounced than that on the 40% MC group. Furthermore, isometric contraction exercise at 90% MC did not facilitate recovery as a function of maximal isometric torque or myofiber CSA. Isometric contraction at 60% MC could not be repeated more than 30 times at ≥90% of torque at the onset, and isometric contraction exercise at 90% MC could not be repeated more than 3 times at the same torque as that at the onset (Figs. 1C-F). In contrast, isometric contraction at 40% MC could be repeated more than 50 times without a decrease in the initial torque. Evidence indicates that resistance training at high intensities that cannot be repeated more than 8–12 times has a large hypertrophic effect on normal human muscles [30,31]. Thus, resistance training at a lower intensity than

that typically performed for normal muscle may be sufficient to facilitate recovery of muscle strength and myofiber CSA of atrophied mouse muscle.

To determine why isometric contraction exercise at 90% MC reduced recovery of maximal isometric torque or myofiber CSA compared with that at 40% and 60% MC, we evaluated the distribution of myofiber CSAs in each muscle (Fig. 5B, D). We found more myofibers with smaller CSAs (<500 μm^2) in muscles trained at 60% or 90% MC compared with muscles of the other groups of mice. After 3 days of isometric contraction exercise at 60% or 90% MC, muscles contained dMHC-positive myofibers (Fig. 6B), a characteristic of regenerated myofibers [54-56]. Moreover, after 7 days of isometric contraction exercise at 90% MC, muscles contained fibers with a central nucleus (Fig. 6D, E). In general, such fibers are frequently observed in damaged muscle [57,58]. The muscles should be composed of a mixture of fibers at different stages of recovery, including atrophied, recovered, damaged, and newly formed (regenerated) fibers either on DAY3 or DAY7 of the isometric contraction exercise in our study. Although we could not identify the recovery status of each fiber due to technical difficulties, we can see the myofibers with infiltrating monocytes and the empty area in the image of muscles trained at 60% (Fig. 6Ac) and 90% MC (Fig. 6Ad) on DAY3 and 90% MC (Fig. 6Bd and Cd) on DAY7, suggesting that the muscle could be damaged by isometric contraction exercise at these intensities. Although the percentage of fibers with a central nucleus in this study seems to be low (~2%) compared with that of drug-induced muscle damage (~30%) [59], Smith et al[60] have reported that

fibers with a central nucleus were observed at ~2.5% on day 7 after downhill treadmill exercise in rat muscle. Furthermore, because the extent of damaged fibers is not a one-to-one correspondence with that of newly formed fibers, and hence central nucleated myofibers, the dissociation of extents between potentially damaged myofibers in Fig. 6A, C and central nucleated myofibers (Fig. 6E) in our study may not be contradicted.

Potentially damaged myofibers with infiltrating mononuclear cells were observed in muscles trained at 60% and 90% MC (Fig. 6A). However, isometric contraction exercise at 60% MC facilitated recovery of the maximal isometric torque and CSA. The myofiber CSAs of muscles trained at 60% MC were smaller than those of muscles trained at 40% MC, but were larger than those of the groups trained at 10% and 90% MC or that of the NT group. The distribution of the myofiber CSAs of muscles trained at 60% MC was similar to those of muscles trained at 40% MC. Thus, a similar mechanism that facilitates recovery from muscle atrophy may account for 60% and 40% MC, although the mean myofiber CSA was smaller at 60% MC due to expression of small regenerated myofibers due to recovery from exercise-induced muscle damage.

It should be noted that the muscle weight of 90% MC mice was not different, and even heavier, from that of CON mice at both DAY3 and DAY7 after isometric contraction exercise (Table 1). It has been reported that repeated bouts of eccentric exercise without appropriate rest intervals induces edema in muscle.[61]. This observation suggests that the relatively heavier muscle weight of 90% MC

mice might be due to edema induced by daily resistance exercise at relatively high intensity (90% MC) for the atrophied muscle.

Myofibers with small diameters were observed equally in muscles trained at 90% and 60% MC; however, myofibers with CSA $\geq$ 2500 μm^2 were observed in muscles trained at 40% and 60% MC, and in muscles of the CON group, they were observed infrequently in the muscles trained at 90% MC. Muscle damage incurred during high-intensity exercise is a hallmark of hypertrophy of normal muscle [62-64] . However, other reports claim that muscle damage is not necessarily required for hypertrophy [65-68]. Our findings indicate that recovery from muscle atrophy was not maximized if muscles were trained at intensities that resulted in muscle damage (60% or 90% MC). This suggests that resistance training should be performed at maximal intensity without muscle damage to achieve recovery of the atrophied muscle. Further studies will be required to elucidate whether a different mode of resistance training such as the combined resistance protocol[52] at non-damaging intensity would enhance the recovery of atrophied muscle.

4-2. Differences in the numbers of myonuclei generated during isometric contraction exercise at varying intensities

There is a positive correlation between myofiber size and myonuclei number [14]. Therefore, an increase in the number of myonuclei is considered important for increase in myofiber CSA. When we

determined the number of myonuclei in muscles trained at different intensities for 7 days, we found that the number of myonuclei and CSAs of microfibers were not increased by isometric contraction exercise at 10% MC (Fig. 6F). In contrast, isometric contraction exercise at 40% MC increased the number of myonuclei more than that of the CON group. Myogenic satellite cell activation, proliferation, differentiation, and fusion with existing myofibers increase in the number of myonuclei[69,19]. Moreover, myogenic satellite cells were activated by isometric contraction exercise at 40% MC (Figs. 7A, B).

We demonstrated that stand-up exercise training performed by mice with atrophied muscles increases the number of myonuclei above normal levels through fusion of myogenic satellite cells with myofibers before recovery of myofiber CSA [34]. A recent study found that the number of myonuclei does not increase during recovery of myofiber size by reloading after TS, although this study assessed only 'spontaneous recovery' from muscle atrophy simply from reloading [70]. Considering that the load applied to the ankle joint during reloading is equivalent to that applied while walking on a flat surface, this corresponds to isometric contraction exercise at 10% MC (~1 mNm), which in our study did not increase the number of myonuclei. Therefore, a significant increase in the number of myonuclei induced by isometric contraction exercise at 40% MC may facilitate the recovery of myofiber CSA. Some studies indicated that myogenic satellite cell proliferation/differentiation are promoted by mechanical stimulation. Myogenic satellite cells on cultured myofibers have been reported to be

activated by mechanical stretch[71] or growth signals[72], and mechanically activated satellite cells could differentiate and proliferate myofibers *in vitro*[73]. On the other hand, myogenic satellite cells can be activated by proinflammatory cytokine during the regeneration of myofibers following muscle damage[74-79]. These data have been used for evidence that muscle damage is needed for the hypertrophy in normal muscle[57,55,56]. However in this study, isometric contraction exercise at 40% MC activated myogenic satellite cells and increased myonuclei without muscle damage, suggesting that the mechanisms other than inflammatory factors may operate the effect of isometric contraction exercise. Further studies will be required to elucidate this unknown mechanisms that related to myogenic satellite cells.

Although isometric contraction exercise at 90% MC increased the number of myonuclei beyond CON levels and activated myogenic satellite cells (Fig. 7A, B), the increase in the number of myonuclei seemed smaller than that at 40% and 60% MC (Fig. 6F). The numbers of Pax7+ nuclei of the 40%, 60%, and 90% MC groups were higher than in the 10% MC and CON groups (Fig. 7A), and no significant difference was observed among the 3 MC groups. This result suggests that satellite cells of the 90% MC group might have proliferated to the same degree as that of the 40% and 60% MC groups. However, some of the cells died and were eliminated, perhaps as a result of the harsh environment associated with muscle damage and fewer survived as compared to that of 40% and 60% MC where the environment was presumably less harsh without damage. If the harsh conditions

eliminated the proliferated satellite cells, the recovery-facilitating effect of the isometric contraction exercise would not simply be a function of times per week (i.e., isometric contraction exercise at 90% MC every 2 days would be equivalent to that at 40% MC everyday), and the less harsh intensity is more important than the adequate total amount of the work for the recovery of atrophied muscle. Furthermore, a portion of the activated myogenic satellite cells of the 90% MC group would have differentiated into new myofibers. The isometric contraction exercise at 90% MC did not therefore increase the number of myonuclei to the same extent as 40% or 60% MC. The presence of fewer myonuclei at 90% MC may have caused a marginal increase in myofiber CSA.

This study shows that different intensities of isometric contraction exercise in mice with atrophied muscles caused varied responses in the same manner as in normal muscle (Table 2.). However, isometric contraction exercise at a non-damaging intensity facilitated recovery from muscle atrophy during an early phase of recovery. Therefore, our method may facilitate development of effective training for rehabilitating atrophied muscle and illuminating the mechanisms of recovery from muscle atrophy.

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Table 1. Body weights and wet weights of soleus muscle

	NT	10% MC	40% MC	60% MC	90% MC	CON
Body weight (g)						
DAY 3	38.1±1.4†	37.3±1.6†	39.1±1.0	39.5±1.1	36.5±2.2†	41.0±1.1*
DAY 7	44.2±1.9	43.2±3.3	42.3±1.9†	43.7±2.5	43.1±3.0	46.5±1.6
Soleus muscle weight (mg)						
DAY 3	10.0±1.1†	10.0±0.6†	10.2±1.2†	10.2±1.0†	10.4±1.6†	12.5±1.1*
DAY 7	10.6±0.7†	11.0±1.1†	12.6±1.1*	11.7±0.8	12.6±1.8*	12.9±0.7*
Soleus muscle weight /body weight (mg/g)						
DAY 3	0.26±0.02†	0.26±0.02	0.25±0.02†	0.25±0.02†	0.28±0.03	0.30±0.02*
DAY 7	0.24±0.02†	0.26±0.02	0.30±0.02*	0.27±0.02	0.30±0.03*	0.28±0.01*

Data are presented as the mean ± standard error of the mean (SEM). n/group = 6. *$P < 0.05$ vs the NT group, † $P < 0.05$ vs the CON group at each time.

Table 2. Conclusion of results in this study

	10%MC	40%MC	60%MC	90%MC
Maximal torque	—	↑	↑	—
Myofiber CSA	—	↑↑	↑	↑
Damaged myofibers	±	±	+	++
Nascent myofibers	±	±	+	++
No. of myonuclei	—	↑↑	↑	↑
Myogenic satellite cell activation	±	+	+	+
The facilitating effects of recovery from atrophy	—	++	+	±

Results of this study are summarized schematically. ↑increase, –no change, +exist.

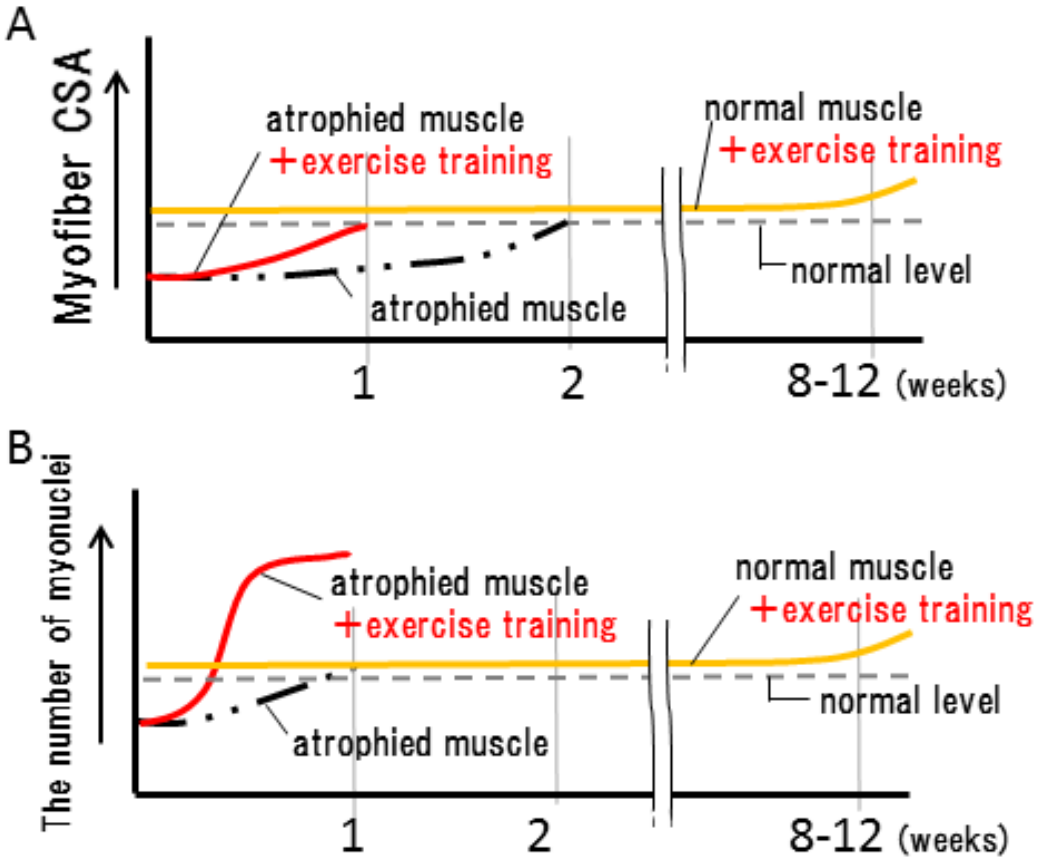
A
Myofiber CSA
atrophied muscle
+exercise training
normal muscle
+exercise training
normal level
atrophied muscle
1
2
8-12 (weeks)
B
The number of myonuclei
atrophied muscle
+exercise training
normal muscle
+exercise training
normal level
atrophied muscle
1
2
8-12 (weeks)

Fig. 1. Effects of resistance training on the myofiber CSA and myonuclus number.

(**A**) When the mice were fed normally after tail suspension, their mean myofiber CSA did not recover to the level of CON group by 7 days. In contrast, the mean myofiber CSA of mice that were subjected to resistance training recovered to nearly the same level of CON group within 7 days. The short-term exercise training on normal muscle require 8–16 weeks for muscle hypertrophy, this period is quite longer than that of the exercise-facilitated recovery of atrophied muscle. (**B**) The number of myonuclei in atrophied muscles started to increase as early as on day 4 after the onset of resistance training, suggesting that the increase in the myonuclei number in atrophied muscle induced by resistance training occurs much earlier than in the exercise induced hypertrophy in normal muscle.

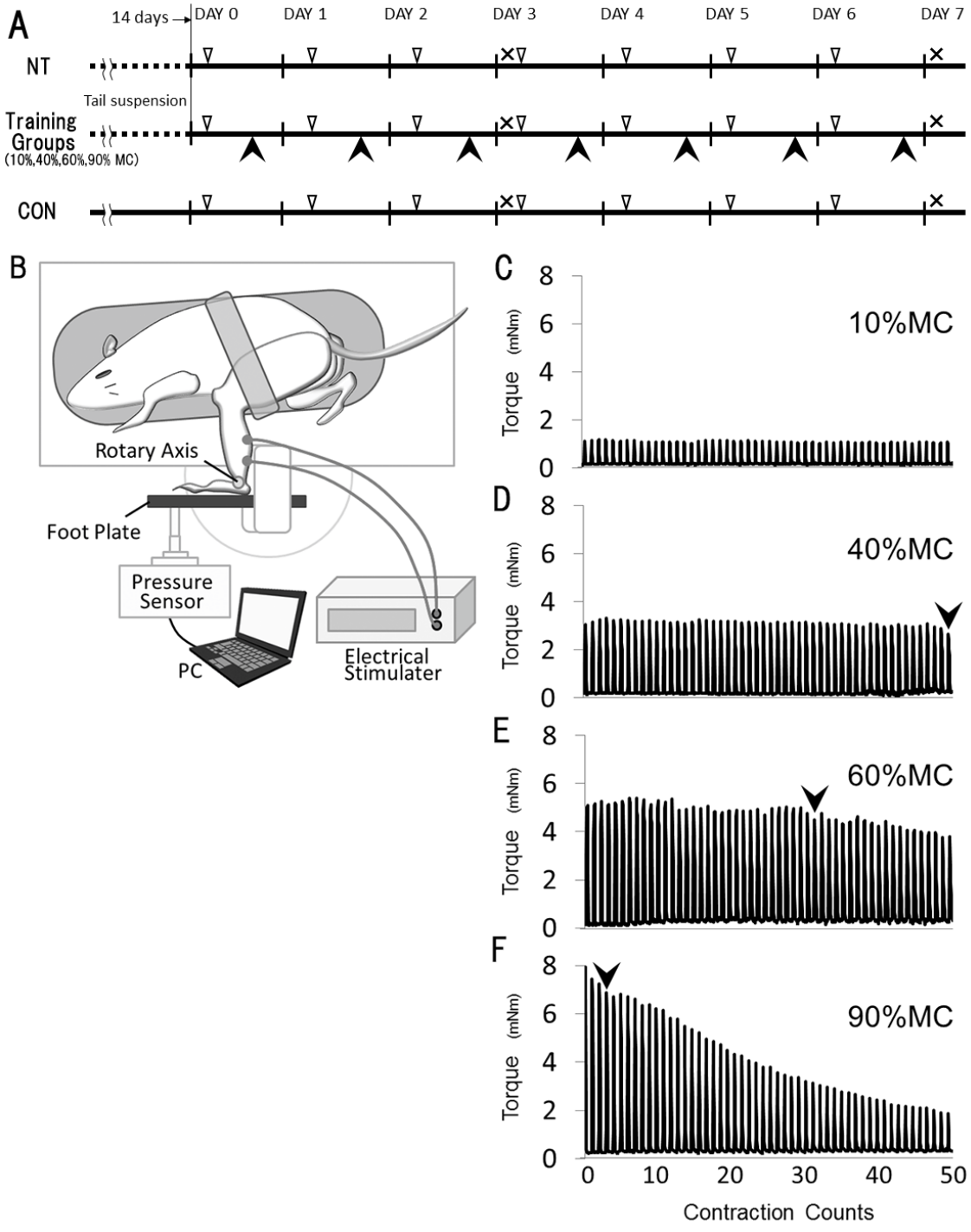
A
14 days
DAY 0
DAY 1
DAY 2
DAY 3
DAY 4
DAY 5
DAY 6
DAY 7
NT
Tail suspension
Training Groups
(10%,40%,60%,90% MC)
CON
B
Rotary Axis
Foot Plate
Pressure Sensor
PC
Electrical Stimulater
C
10%MC
D
40%MC
E
60%MC
F
90%MC
Torque (mNm)
0
2
4
6
8
0
10
20
30
40
50
Contraction Counts

Fig. 2. Scheme for intervention times and method of quantitative muscle contraction. (A) Experimental protocol for animals. Open triangles, functional analysis; filled arrowheads, isometric contraction exercise (50 repetitions /day); X marks, histological analysis. **(B)** Diagram of the device used for isometric muscle contraction. The device consisted of an electrical stimulator and a pressure sensor. Electrical stimulation was administered to the posterior surface of the skin of the lower limbs. Plantar flexion torque was calculated from the pressure added to the footplate. (**C–F)** Torques at the onset of contraction were set to 1.0, 3.0, 5.0, and 8.0 mNm, representing 10%, 40%, 60%, and 90% MC strength for each group, respectively. An ~10% decrease in torque was observed after 50, 30, or 2 repetitions in 40%, 60%, and 90% MC groups, respectively (**D-F,** arrowheads).

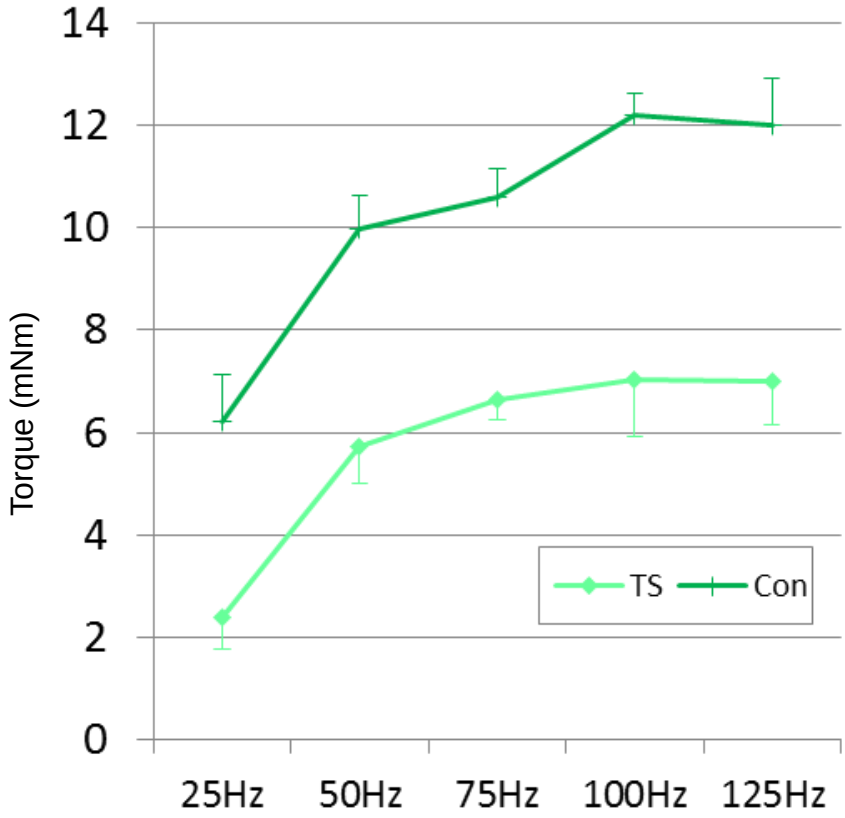
14
12
10
8
6
4
2
0
Torque (mNm)
25Hz
50Hz
75Hz
100Hz
125Hz
TS
Con

Fig. 3 The torque evoked by electrical stimuli with a respective frequency. The torque were measured by exposing muscle to electrical stimulus at successive frequencies (25, 50, 75, 100 and 125 Hz) in the mice following tail suspension (TS) and normal mice (CON). At 100 Hz, the maximal torque was obtained, and reached a plateau.

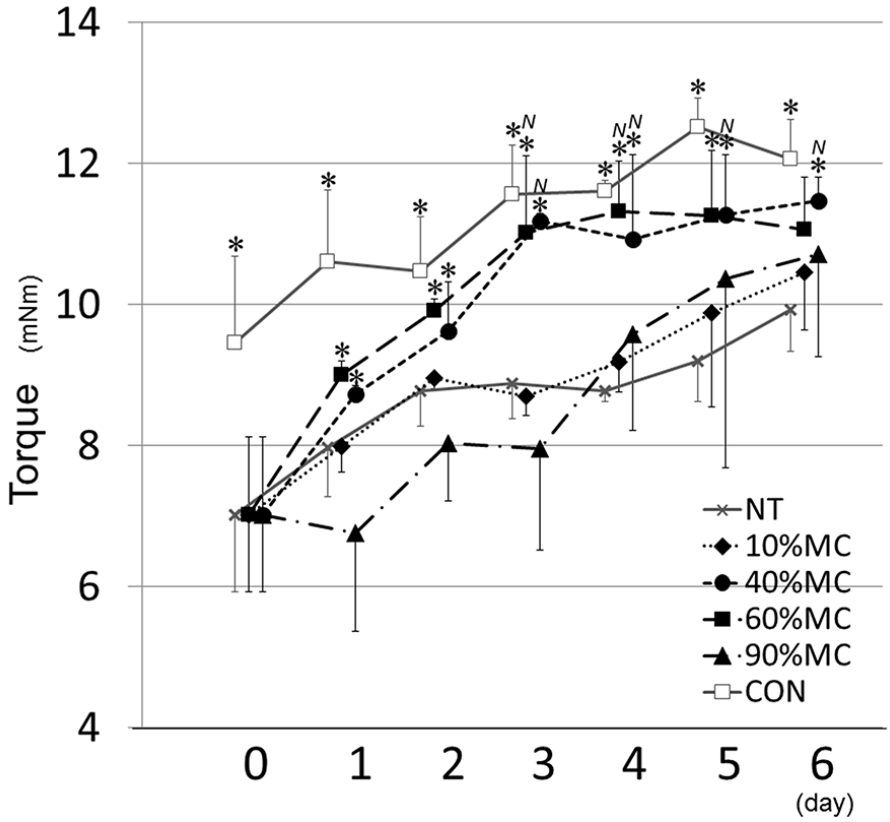
14
12
10
8
6
4
Torque (mNm)
0
1
2
3
4
5
6
(day)
NT
10%MC
40%MC
60%MC
90%MC
CON

Fig. 4. Recovery of maximal isometric torque of the atrophied muscle by isometric contraction exercise at varying intensities. The values of maximum isometric torque were significantly greater in the 40% and 60% MC groups than in the NT group during 1–4 days (during the DAY1-4) of isometric contraction exercise. Values of maximum isometric torque in the 40% and 60% MC groups were approximately the same as that of the CON group after 3 days of training (on DAY3). Results represent the mean ± SEM; n/group = 6. *$P < 0.05$ vs NT group at each time, N, not significant from the CON group.

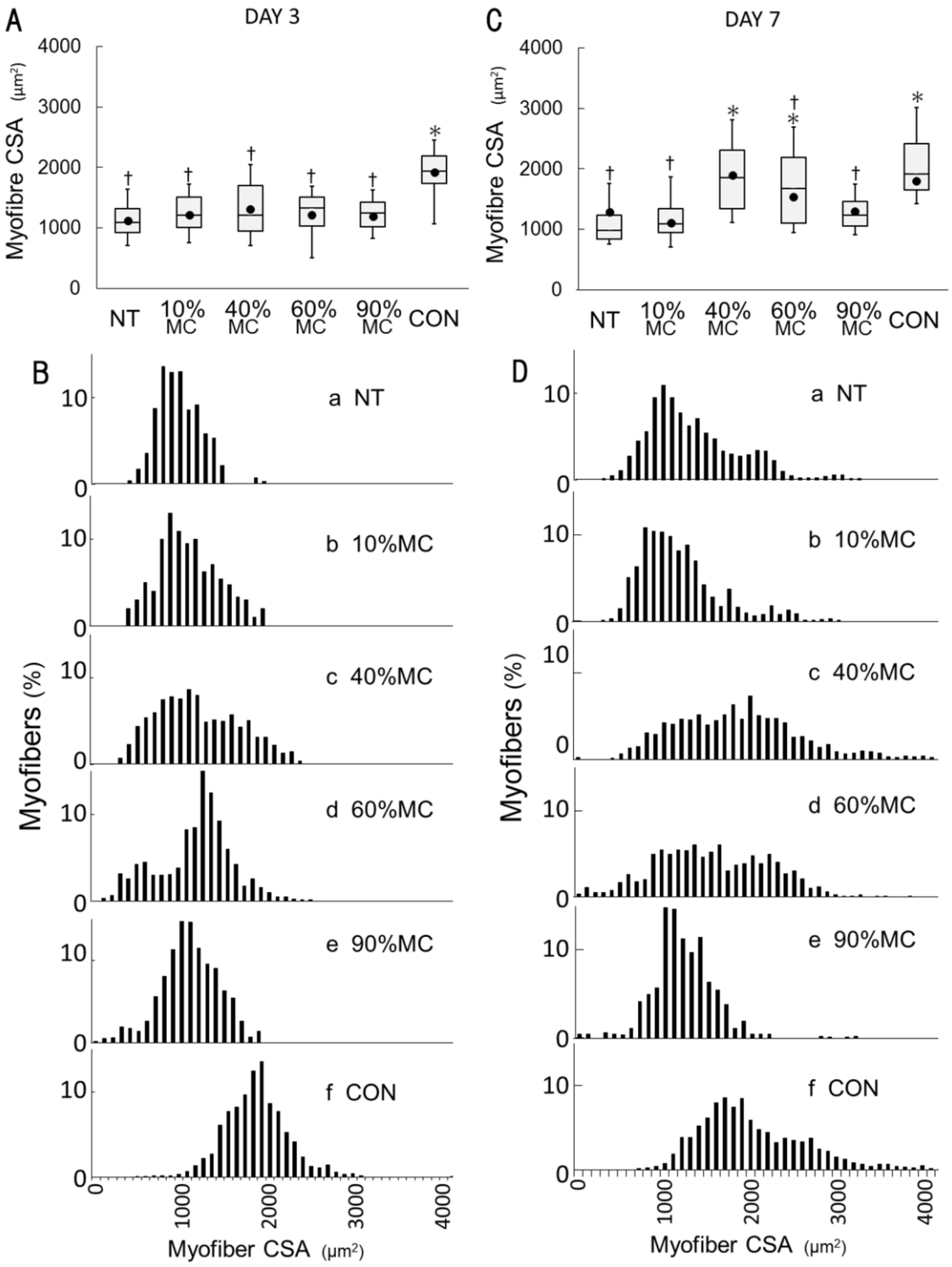
A
DAY 3
Myofibre CSA (μm²)
4000
3000
2000
1000
0
NT
10% MC
40% MC
60% MC
90% MC
CON
C
DAY 7
Myofibre CSA (μm²)
B
a NT
b 10%MC
c 40%MC
d 60%MC
e 90%MC
f CON
Myofibers (%)
10
0
1000
2000
3000
4000
Myofiber CSA (μm²)
D
a NT
b 10%MC
c 40%MC
d 60%MC
e 90%MC
f CON
Myofibers (%)
Myofiber CSA (μm²)

Fig. 5. Changes in muscle histology during the recovery of atrophied muscle that occurred during isometric contraction exercise at various intensities. (A, C) Box plots show the Standard Deviation (SD) of the myofiber CSA of each group 3 (on DAY3, **A**) or 7 days (on DAY7, **C**) after the isometric contraction exercise following TS. Dots indicate mean values. Boxes indicate the twenty-fifth and seventy-fifth percentiles. Horizontal lines in the boxes indicate median values. Vertical lines denote tenth and ninetieth percentiles, respectively. n; fibers/group = 3426-4259. $^{*}P < 0.05$ vs the NT group, $^{\dagger}P < 0.05$ vs the CON group. **(B, D)** Myofiber CSAs of each group on days 3 (**B**) or 7 (**D**) after training.

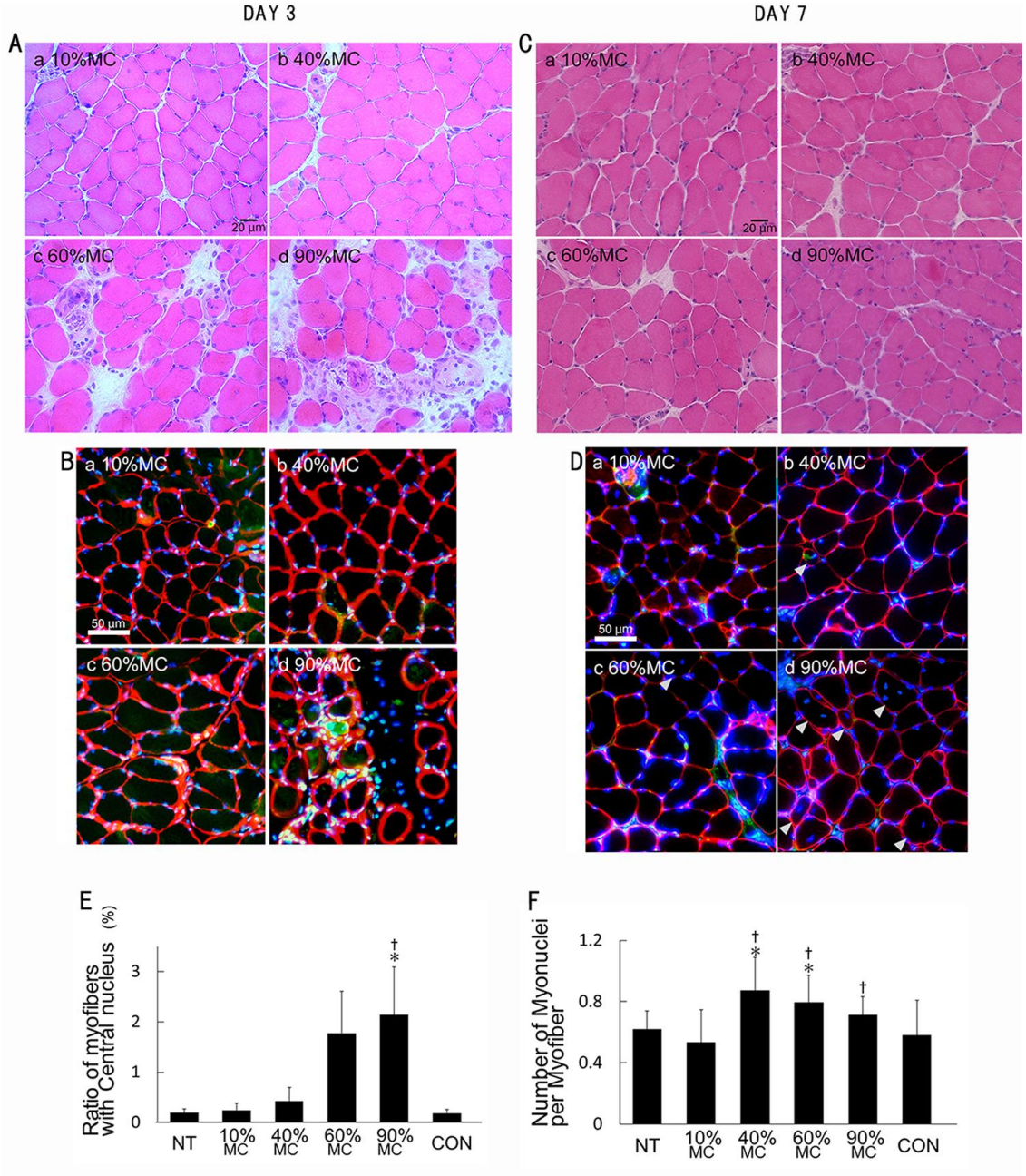
DAY 3
DAY 7
A
a 10%MC
b 40%MC
c 60%MC
d 90%MC
20 μm
C
a 10%MC
b 40%MC
c 60%MC
d 90%MC
20 μm
B
a 10%MC
b 40%MC
c 60%MC
d 90%MC
50 μm
D
a 10%MC
b 40%MC
c 60%MC
d 90%MC
50 μm
E
Ratio of myofibers with Central nucleus (%)
0
1
2
3
NT
10% MC
40% MC
60% MC
90% MC
CON
F
Number of Myonuclei per Myofiber
0
0.4
0.8
1.2
NT
10% MC
40% MC
60% MC
90% MC
CON

Fig. 6. Induction of muscle damage and increase in the number of myonuclei in atrophied muscle caused by isometric contraction exercise at various intensities. Photomicrographs of muscle samples 3 or 7 days after the training stained by hematoxylin–eosin (**A, C**), and immunohistochemistry with anti-developmental myosin heavy chain (green), anti-dystrophin (red) and DAPI (blue) (**B, D**). Damaged or regenerated fibers or both were observed notably in the 60% and 90% MC groups. Arrows, dMHC-positive myofibers, Arrowheads, central nucleated myofibers. (**E)** The ratio of myofibers with central nuclei from mice trained for 7 days following TS (DAY7). Results represent the mean ± SEM; n; fibers/group = 2259-3589. (**F**) The number of myonuclei per myofiber from trained muscle for 7 days following TS (DAY7). Results represent the mean ± SEM; n; fibers/group = 4742-6732. *$P <$ 0.05 vs the NT group, $^{\dagger}P < 0.05$ vs the CON group.

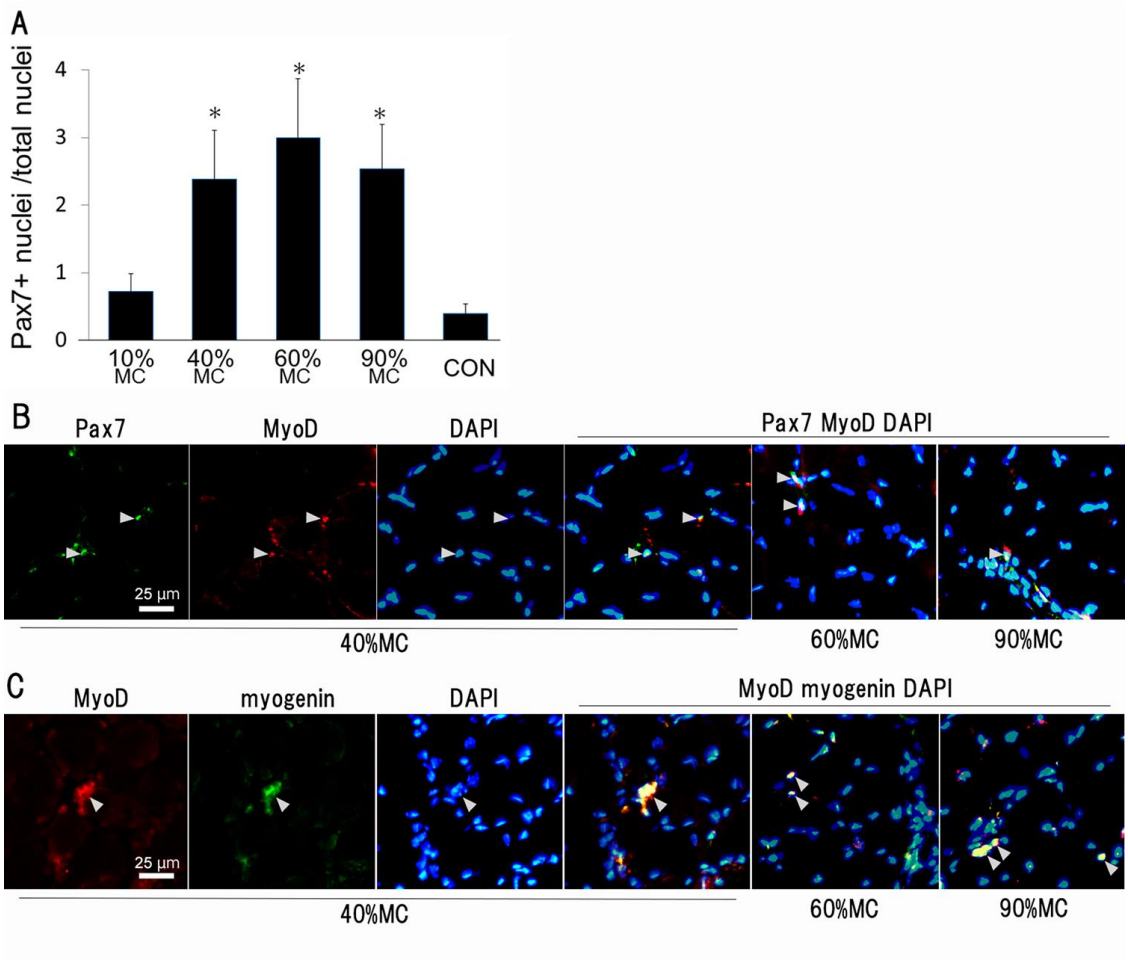
A
Pax7+ nuclei /total nuclei
4
3
2
1
0
*
*
*
10% MC
40% MC
60% MC
90% MC
CON
B
Pax7
MyoD
DAPI
Pax7 MyoD DAPI
25 μm
40%MC
60%MC
90%MC
C
MyoD
myogenin
DAPI
MyoD myogenin DAPI
25 μm
40%MC
60%MC
90%MC

Fig. 7. Activation of myogenic satellite cells during recovery of atrophied muscle induced by isometric contraction exercise at various intensities. (A) The number of Pax7 positive nuclei per total nuclei from trained muscle for 7 days following TS (DAY7). The numbers of Pax7 positive nuclei were significantly higher in the 40% MC, 60% MC, and 90% MC groups than in the CON group. Results represent the mean ± SEM; n; nuclei/group = 58667-78201. *$P < 0.05$ vs the CON group. Typical sections probed with anti-MyoD, anti-Pax7 **(B)** or anti-MyoD, anti-Myogenin antibodies **(C)** from mice of the 40%, 60%, and 90% MC groups. The nuclei that expressed MyoD or Myogenin were immunoreactive with antibodies against Pax7 or MyoD (arrowheads).

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和文抄録

Training at non-damaging intensities facilitates recovery from muscle atrophy

（筋萎縮からの回復促進には筋損傷の起こらない程度の筋力トレーニングが有効である

―マウス萎縮筋に対する等尺性筋力トレーニングによる検証―）

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筋萎縮からの回復を促すことを目的に筋力トレーニングが行われるが，萎縮筋に対してどれぐらいの強度で運動すれば最も効果的であるのか十分に解明されていない．そこで本研究は，萎縮筋に対する筋力トレーニングの強度と筋萎縮からの回促進効果との関係を調べた．2週間の尾部懸垂により萎縮したマウスの足関節底屈筋群に対して，収縮強度の異なる等尺性筋収縮運動をくりかえし行わせた．等尺性筋収縮運動は，麻酔下のマウス下腿後面に電気刺激を50回/日，7日間与えることで行った．収縮強度は与える電気刺激の電流値を変えることにより調整し，刺激１回目に発揮される足関節底屈トルクを最大等尺性収縮トルクの10, 40, 60, 90%に設定した（各々10%, 40%, 60%, 90% MC群、各n=12）．その結果，40%, 60% MC群では最大等尺性収縮トルクや筋線維横断面積の回復が，尾部懸垂後運動を行わず通常飼育した群（NT群）に比べて促進していることが判明した．一方，60, 90% MC群の筋には，損傷した筋線維や再生過程にある筋線維が認められた．40%, 60%, 90% MC群の筋には，10% MC群やNT群には見られなかった筋線維核の正常以上の増加とともに筋衛星細胞の活性化が見られた．この現象は運動刺激によって活性化された筋衛星細胞が筋線維へと融合することで筋線維核を増やし筋線維サイズの回復を図った結果だと考える．本研究の結果から，筋萎縮からの回復促進のためのトレーニングには，損傷を起こさない程度の適度な強度の運動が有効であると考える．