J. Phys. Ther. Sci. 35: 193–198, 2023

The Journal of Physical Therapy Science

Original Article

Division of loading time in reloading the disused atrophic soleus muscle induces proximal muscle injury

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Abstract. [Purpose] This study aimed to compare the effects of loading time division in reloading atrophied muscles in different muscle long-axis regions. [Materials and Methods] We divided 8-week-old male Wistar rats into control (CON), 14-day hindlimb suspension (HS), 7-day hindlimb suspension followed by 60-min reloading for 7 consecutive days (WO), and 7-day hindlimb suspension followed by 60-min reloading on two separate occasions for 7 days (WT) groups. After the experimental period, muscle fibre cross-sectional area and necrotic fibre/central nuclei fibre ratio were measured in the soleus muscle's proximal, middle, and distal regions. [Results] The necrotic fibre/central nuclei fibre ratio was higher in the WT group than in the other groups in the proximal region. Proximal muscle fibre cross-sectional area was higher in the CON group than in the other groups. In the middle region, only HS group had muscle fibre cross-sectional area lower than the CON group. Similarly, muscle fibre cross-sectional area of the HS group was lower than the CON and WT groups in the distal region. [Conclusion] When reloading atrophied muscles, dividing the loading time can inhibit atrophy in the distal region but induce muscle injury in the proximal region.

Key words: Reloading, Muscle atrophy, Muscle injury

(This article was submitted Oct. 20, 2022, and was accepted Dec. 1, 2022)

INTRODUCTION

Muscle is an adaptive organ, atrophying with disuse and hypertrophying with training. Disuse muscle atrophy occurs locally or systemically when muscle synthesis and breakdown are out of balance due to muscle disuse. Examples include cast immobilization due to fracture, bed rest, and when muscle synthesis is greater than breakdown[1–3)]. Muscle atrophy is a factor that interferes with daily living activities; the associated decrease in activity leads to further muscle atrophy and secondary problems[3–6)]. Differing from neuromuscular disease muscle atrophy, the muscles atrophied from disuse are more likely to respond to training, such as reloading and resistance exercises[7–9)]. Therefore, it is recommended that training be implemented early in physiotherapy for the disused atrophic muscles[9)].

Compared to normal muscles, atrophic muscles require different conditions when training for muscle mass changes. For normal muscles, a higher recommended load, number of repetitions, number of sets, and training frequency, as well as shorter rest times, can be used to achieve the hypertrophic effect[10–13)]. However, since atrophic muscles are more fragile[14, 15)], excessive muscle damage may occur if the same load is applied as in the normal muscles. Excessive muscle damage interferes with subsequent training due to delayed onset muscle pain[3)] and may also directly lead to muscle fibre atrophy and muscle

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weakness[16]. Therefore, when training atrophic muscles, it is necessary to set conditions that facilitate changes in muscle mass whilst considering muscle damage. It has been reported that muscle hypertrophy is more effective in a 3-h rest period than in a 1-h rest period when the same load is applied to the muscle[17]. Therefore, a more significant inhibitory effect on muscle atrophy (recovery) can be obtained by adding a specific rest period when loading, including gradual reloading to the atrophic muscles. It is also possible that dividing the loading time may reset the accumulation of stress on the atrophic muscles and lead to load dispersion.

In addition, the structure of muscles differs in long-axis regions; thus, it is necessary to consider these regions when training against muscle atrophy. Properties, such as muscle extensibility and fibre type composition, are not uniform in the muscle's long-axis regions; different long-axis regions assume different functions[18, 19]. Therefore, when reloading atrophic muscles, it is expected that the amount of load applied and the load response may differ depending on the long-axis region. Several studies have reported that the progression of disused muscle atrophy, due to unweighting and training changes, differs in the long-axis region of the same muscle[20–22]. Muscle damage does not occur uniformly in the long-axis region; despite an absence of damage in the middle region of the muscle belly, there may be areas where damage occurs when the long-axis region is verified. Hence, verification at the long-axis region of the muscle is necessary to evaluate muscle condition.

There are no reports examining the effect of loading time division on the reloading of atrophic muscles at the long-axis regions. Therefore, this study aimed to compare the effects of dividing the loading time on the atrophic muscles for different long-axis regions to help set the loading conditions on the atrophic muscles.

MATERIALS AND METHODS

The subjects were 24 8-week-old male Wistar rats weighing 250.6 ± 41.2 g. They were divided into (1) a normally-reared group (control [CON] group; n=4); (2) a hindlimb suspension group (HS group; n=6) with hindlimb suspension for 14 days; (3) a once weight-bearing group (WO group; n=7) with hindlimb suspension for 7 days followed by reloading once a day; and (4) a twice weight-bearing group (WT group: n=7) with hindlimb suspension for 7 days followed by reloading twice a day. Rats were kept in an environment with free access to water and food during the experiment. The room temperature was maintained at 24.0 ± 2.0 °C with a 12-h light-dark cycle.

This study was conducted with the approval of the Kanazawa University Animal Experiment Committee (AP-163790).

For hindlimb suspension, rats were attached to a hindlimb suspension brace, and the hindlimbs were unloaded by suspending the dorsal pelvic region upward, referring to previous studies[20, 21]. The rats could move around the cage using their forelimbs even when their hindlimbs were suspended.

In the WO/WT groups, the suspension device was removed for the specified loading time, and the limbs were grounded to the floor. The total loading time was 60 min/day for both the groups. The WO group was loaded once for 60 min continuously, and the WT group was loaded twice for a total of 60 min, divided into two 30-min periods with a 4-h rest period in between. The WO and WT groups continued hindlimb suspension during the 7-day reloading period except for the loading time.

At the end of the 14-day experimental period, the weight was determined, and the left soleus muscle was removed under intraperitoneal anaesthesia. The excised muscle was quickly frozen in isopentane cooled using liquid nitrogen immediately after wet weight determination. The muscle was then divided 25% (proximal), 50% (middle), and 75% (distal) of the distance away from the origin. The proximal, middle, and distal sections of 10 µm thickness were prepared using a cryostat (Sakura Finetek CRY03, Tokyo, Japan). Hematoxylin-eosin staining was performed on the prepared sections, and microscopic images were analyzed (Fig. 1). Muscle fibre cross-sectional area (MCSA) was measured using the image analysis software, Image J. The MCSA was measured for more than 100 muscle fibres in the centre and surrounding areas of each section that did not overlap, and the mean value of the MCSA was calculated. The number of necrotic fibres indicated the degree of muscle damage, and the number of central nuclei fibres with central nuclei indicated the degree of muscle regeneration during the muscle recovery process[9, 23]. Necrotic fibres were defined to contain phagocytic infiltration or marked loss of staining (Fig. 2a), and the necrotic fibre ratio (number of necrotic fibres/total muscle fibres × 100) was calculated. The central nuclei fibre ratio (number of central nuclei fibres/total muscle fibres × 100) was also calculated for the central nuclei fibres (Fig. 2b).

Statistical package for the social sciences version 28 (IBM SPSS Statistics, Japan IBM, Tokyo, Japan) was used for statistical analysis. One-way analysis of variance and Tukey's multiple comparisons were used to compare the groups, with a significance level of 5%. The results were presented as mean ± standard deviation.

RESULTS

Body weight, soleus muscle wet weight, and relative weight ratio (muscle wet weight/body weight) for each group are shown in Table 1. Body weight and wet muscle weight were significantly higher in the CON group (body weight 329.8 ± 12.7 g, wet muscle weight 114.0 ± 4.2 mg) than in the HS (body weight 212.5 ± 20.5 g, wet muscle weight 59.2 ± 5.8 mg), WO (body weight 219.4 ± 4.9 g, wet muscle weight 71.4 ± 12.3 mg), and WT (body weight 232.1 ± 16.8 g, wet muscle weight 74.7 ± 12.5 mg) groups (Table 1). Relative weight ratios were not significantly different between the groups (Table 1).

MCSA of each group is shown in Table 2. The proximal MCSAs were 48.2%, 62.1%, and 65.9% in the HS (1,210.4 ± 207.5 µm^2), WO (1,560.6 ± 369.6 µm^2), and WT (1,656.5 ± 416.1 µm^2) groups, respectively. The CON group had a

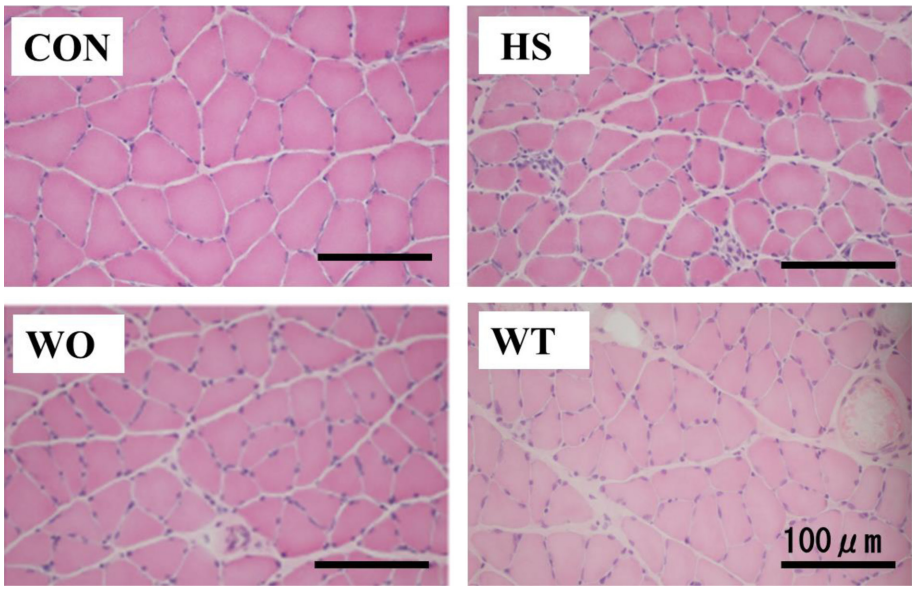

Fig. 1. Representative hematoxylin-stained images of each group.
CON: control group; HS: hindlimb suspension group; WO: once weight-bearing group; WT: twice weight-bearing twice group.
Hematoxylin-eosin stained image, original magnification 200×.

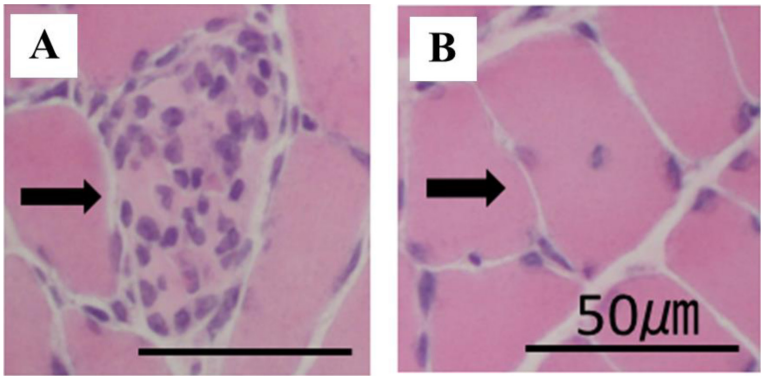

Fig. 2. Light microscopy images demonstrationg A: necrotic fibres, B: central nuclei fibres.
Hematoxylin-eosin stained image, original magnification 200×.

significantly higher MCSA than the other groups (2,513.5 ± 214.2 μm^2) (Table 2). The HS group's middle region MCSA was 46.2% (1,219.1 ± 268.1 μm^2), which was significantly lower than of the CON group (Table 2). Conversely, there was no significant difference between the CON, WO, and WT groups. The distal MCSA of the HS group (1,287.3 ± 222.7 μm^2) was significantly lower than that of the CON (51.8%, 2,486.6 ± 295.8 μm^2) and WT groups (64.8%, 1,987.6 ± 518.6 μm^2) (Table 2).

The necrotic fibre ratio was not significantly different between the groups in any region. The proximal region central nuclei fibre ratio was significantly higher in the WT group (3.1 ± 1.9%) than in the CON (0.5 ± 0.6%), HS (0.3 ± 0.4%), and WO groups (0.9 ± 1.0%) (Table 3).

DISCUSSION

This study compared the influence of dividing the loading time on MCSA and muscle damage when reloading disused atrophic muscle in different muscle long-axis regions.

The results showed that the HS group had lower MCSA than the CON group in all regions; this suggests hindlimb suspension caused muscle atrophy in all regions of the long axis of the muscle. Furthermore, the proximal region MCSA was higher in the CON group than in the other groups. However, the HS group was significantly different from the CON group in

Table 1. Comparison of body weight, wet muscle weight, and relative weight ratio for each group

Group	Body weight (g)	Wet muscle weight (mg)	Relative weight ratio (mg/g)
CON (n=4)	329.8 ± 12.7	114.0 ± 4.2	0.3 ± 0.0
HS (n=6)	212.5 ± 20.5*	59.2 ± 5.8*	0.3 ± 0.0
WO (n=7)	219.4 ± 4.9*	71.4 ± 12.3*	0.3 ± 0.1
WT (n=7)	232.1 ± 16.8*	74.7 ± 12.5*	0.3 ± 0.1

Mean ± standard deviation.
*Significant difference from the CON group.
CON: control group; HS: hindlimb suspension group; WO: weight-bearing once group; WT: weight-bearing twice group.

Table 2. Comparison of muscle fibre cross-sectional area in each group

Group	Proximal region (μm^2)	Middle region (μm^2)	Distal region (μm^2)
CON (n=4)	2,513.5 ± 214.2†	2,636.3 ± 228.0	2,486.6 ± 295.8†
HS (n=6)	1,210.4 ± 207.5*	1,219.1 ± 268.1*	1,287.3 ± 222.7*
WO (n=7)	1,560.6 ± 369.6*	1,944.4 ± 621.1	1,805.0 ± 472.6
WT (n=7)	1,656.5 ± 416.1*	1,957.8 ± 546.9	1,987.6 ± 518.6†

Mean ± standard deviation.
*Significantly different from group CON group.
†Significantly different from group HS group.
CON: control group; HS: hindlimb suspension group; WO: weight-bearing once group; WT: weight-bearing twice group.

Table 3. Comparison of necrotic and central nuclei fibre ratios for each group

	Group	Proximal region (%)	Middle region (%)	Distal region (%)
Necrotic fiber ratio	CON (n=4)	0.5 ± 0.6	0.3 ± 0.5	0.5 ± 0.6
	HS (n=6)	1.2 ± 0.9	0.5 ± 0.5	0.9 ± 0.8
	WO (n=7)	1.0 ± 0.8	1.5 ± 1.1	1.8 ± 1.7
	WT (n=7)	1.2 ± 0.9	1.3 ± 1.2	2.3 ± 1.7
Central nuclei fibre ratio	CON (n=4)	0.5 ± 0.6*	0.3 ± 0.6	0.3 ± 0.5
	HS (n=6)	0.3 ± 0.4*	0.7 ± 0.5	0.9 ± 0.7
	WO (n=7)	0.9 ± 1.0*	1.5 ± 1.4	1.5 ± 1.3
	WT (n=7)	3.1 ± 1.9	1.2 ± 0.9	0.9 ± 0.6

Mean ± standard deviation.
*Significantly different from group WT group.
CON: control group; HS: hindlimb suspension group; WO: weight-bearing once group; WT: weight-bearing twice group.

the middle and distal regions, while the WO and WT groups showed no significant differences. Nishikawa et al.[24] reported that the effect of reloading rat soleus muscle after disuse muscle atrophy was more significant in the distal region than in the proximal region. This study also suggested that reloading inhibited muscle atrophy in the middle and distal regions, supporting previous studies. In addition, only the CON and WT groups with twice-daily reloading had significantly higher MCSA than the HS group in the distal region. The simple interpretation of the effect on muscle atrophy inhibition suggests that dividing the loading time into segments is more effective in inhibiting muscle atrophy than continous muscle loading.

Concerning muscle damage, there was no increase in necrotic fibres with loading in this study, but an increase in central nuclei fibres was observed in the WT group. Macrophages are reported to reach their peak 2 days after muscle loading, as temporal changes in muscle repair following muscle injury[25] and necrotic fibres increase[9]. There is a time lag between the generation of necrotic fibres and central nuclei fibres; the number of necrotic fibres decreases with time, and central nuclei fibers are produced as a regenerative response[9]. Therefore, 7 days after initiating reloading, more central nuclei fibres than necrotic fibres were developed in response to muscle injury. This explains why there was a difference between the groups in central nuclei fibres, correlated to loading time but not the number of necrotic fibres. Furthermore, central nuclei fibres were significantly higher in the proximal region of the WT group than in the other groups.

Although previous studies do not agree on the area most damaged by muscle loading, the muscle-tendon transition area[26] and the middle region of the muscle belly[27] have been suggested as the most damaged areas. This difference from our results may be due to variation in target muscles, load type, and muscle conditions. In the rat soleus muscle, it has been reported that atrophy is more likely to progress in the proximal region due to non-loading[20, 24] and the increased fragility of this region. In addition, Cè et al.[18] reported that the distal region of the muscle shows fewer changes in muscle morphology with stretch due to connective tissue, suggesting that the proximal region is more load-bearing than the distal region. These factors may have contributed to the greater proximal muscle damage in this study.

In this study, the impact of loading on muscle damage and the inhibition of muscle atrophy was greater when the loading time was divided, rather than applied continuously. One factor in muscle injury is reduced muscle flexibility[28, 29], and damage occurs when excessive stretching stress is applied to the muscle. In general, hindlimb suspension in rats facilitates plantar flexion of the ankle joint and shortens the ankle plantar flexors[30, 31]. Therefore, muscle damage is likely to occur when reloading due to the application of stretching stress to the shortened soleus muscle at the start of loading. Muscle flexibility gradually improves throughout loading due to muscle contraction and the stretching stimulus caused by loading, making muscle damage less likely. However, when loading twice a day, the ankle plantar flexors return to a less flexible state after the rest period; this is assumed to have caused more load-induced damage. The above results suggest that continuous loading is safer than dividing the loading time when the total loading time is constant. However, since preconditioning, such as warmth and low-intensity exercises, can prevent muscle damage[32–34], divided load training in combination with preconditioning may be more effective for atrophy inhibition.

One limitation of this study was that, although this study was validated on soleus muscle, different muscles may show different results. It has been reported that the degree of change in the long-axis region due to training depends on the muscle[22]. Therefore, it is necessary to investigate the effects of reloading on other muscles and determine the regions that require special attention in different muscles when performing interventions. In addition, this study was analyzed 7 days after reloading, and changes occurring after that time were not investigated. Since the long-term effects of muscle damage on muscle atrophy have not been verified, it is necessary to identify which loading conditions are more effective in reducing muscle atrophy in the long term.

In conclusion, atrophy in the distal muscle is easily suppressed by dividing the reloading time, but this is more likely to induce damage in the proximal region. It is preferable to load continuously to perform load training without muscle damage.

Funding

This research was funded by JSPS KAKENHI Grant Number JP 20K11204.

Conflict of interest

The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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