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The impact of reload after hindlimb suspension of rats

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要旨　目的・方法：廃用性筋萎縮を惹起するためラットを後肢懸垂し，コントロール群（CON），１週間のうち，前３日間のみ後肢懸垂する群（HLS3D），１週間後肢懸垂する群（HLS1W）の計３群に分類し，筋萎縮及び回復の程度を比較した。赤筋と白筋に分け，筋線維短径の変化を比較した。結果：コントロール群と比較して，後肢懸垂群は筋湿重量では全ての筋において，減少傾向が見られた。EDL を除いた２つの筋の筋湿重量は，有意に低下した。EDL は減少傾向を示したが，有意差は見られなかった。筋短径に関しては，SOL のタイプⅠは有意に低下した。しかし，EDL 及び TA のタイプⅡでは低下は認められず，筋肥大を呈する結果となった。また，この２筋のタイプⅠでは，再荷重の影響により HLS3D がもっとも大きな値を示した。

Keyword: hindlimb suspension, muscle atrophy, reload

Abstracts

Background: The disuse muscle atrophy was induced by a hindlimb suspension. This study aimed to investigate the effect of reloading on atrophied muscle. ***Methods:*** Young adult male Wistar rats were reloaded for 4 days after suspension for the first 3 days. ***Results:*** Muscle wet weight of both soleus (SOL) and tibialis anterior (TA) was significantly decreased while one in extensor digitorum longus (EDL) did not reach a significant decrease. The minor axis of type I fibers significantly decreased in SOL. Meanwhile, type II fibers' axis increased in EDL and TA. Four days of reload made the axis of type I fibers the longest in both EDL and TA.

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Introduction

Bed rest after surgery and various diseases are indispensable in the treatment process; however, in some cases, it causes regressive changes to musculature. One of the secondary changes is a muscle atrophy. According to previous studies, it is said that the muscle atrophy rate is the highest in the first week after inactivity[1].

A lot of animal experiments with various models are developed in order to prevent and restore muscle atrophy in an early stage. For example, a hindlimb suspension is the way to evoke muscle atrophy in hindlimbs. In the present study, we examined how quickly the muscle mass was restored by the hindlimb suspension method.

Materials and Methods

Animals

Eight male Wistar rats (347±13 g), 10 weeks of age, were bought from Kyudo, Co, Ltd. The rats were provided one week to acclimate prior to the use for any studies and were fed a regular diet *ad libitim*. They were divided into 3 groups: 1 group was assigned as a control group, and the other 2 groups served as the suspended groups (CONTROL, CON: n=2, hindlimb suspension for 1 week, HLS 1 W: n=3, hindlimb suspension for only the first 3 days, HLS 3 D: n=3). All rats were housed in a 24℃ environment with a light-dark cycle of 12:12 hours. This study was approved by the Institutional Animal Ethics Committee of Nishikyushu University (authorization number: H27-1).

Hindlimb suspension

Rats were suspended in an individual plastic cage. To set and adjust the suspension, rats were briefly anesthetized by inhalation of isoflurane. The end of a thread was attached to the rat's tail with adhesive tape, while another end was connected to a ring, which was attached to a stick suspended from the top of the cage. The rats could move freely on their forelimbs with this treatment. After the rats had fully recovered from the anesthesia, the angle of suspension was adjusted to make sure that when the rats were fully stretched, their hindlimbs were unable to touch the ground. The body weight of the rats was then measured over time during the suspension.

Muscle analysis

Following euthanasia, SOL, TA and EDL were isolated from both hindlimbs. After measuring muscle wet weight, they were formalin-fixed. They were then cut in the muscle belly and paraffin-embedded. These were stained with hematoxylin and eosin (H&E) to visualize tissue morphology, and additionally immunostained with Monoclonal Anti-Myosin (Skeletal, Slow). And then, SOL, TA and EDL were examined under a light microscope (OLYMPUS, BX41) to calculate muscle fiber minor axis. It was determined by using Image J Software (National Institutes of Health, USA).

Statistical analysis

Numerical data were expressed as mean ± standard deviation. Results were analyzed using a one-way analysis of variance (ANOVA). If significance was achieved ($p<0.05$), pairwise comparisons were performed using Tukey-Kramer method.

Results

Body weight

The three groups of rats had similar body weights. The body weight of post-suspension (POST) was decreased significantly at both HLS3D and HLS 1 W when compared to CON (Table 1).

Table 1 (*$p<0.05$)

	CON	HLS3D	HLS1W
PRE	350 ± 10	342 ± 3	348 ± 3
POST	430 ± 5	387 ± 17	370 ± 0
	*		
		*	

Muscle wet weight

As shown on Fig. 1, muscle wet weights of both SOL and TA in HLS1W exhibited a significant decline as compared to CON. EDL with HLS1W showed a significant decrease, compared to CON as well as EDL with HLS3D.

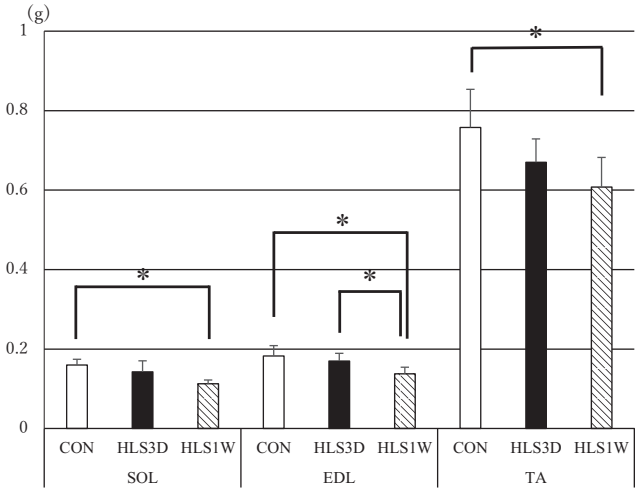

Figure 1 (*$p<0.05$)

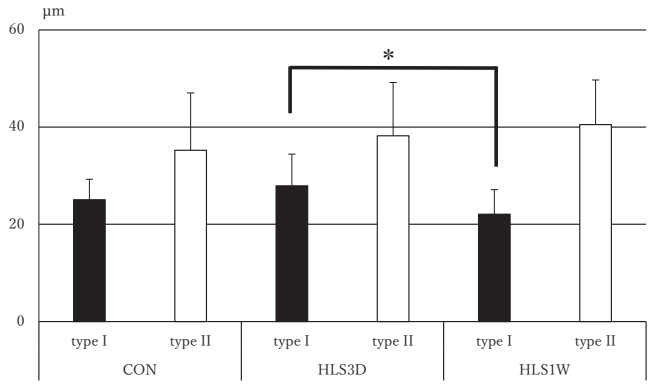

Figure 2 (*$p<0.05$)

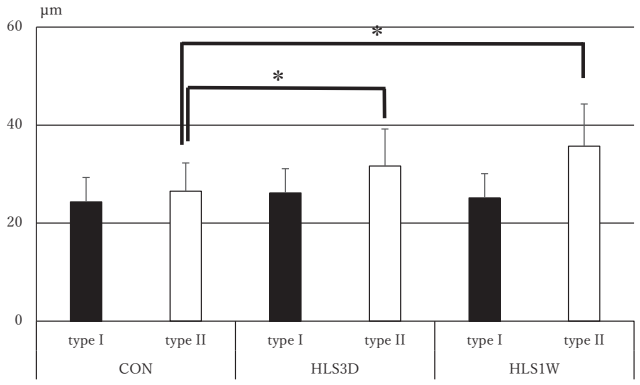

Figure 3 (*$p<0.05$)

Muscle fiber minor axis

Type I fiber axis of EDL was significantly larger in HLS3D than in HLS1W. On the other hand, type II fibers showed no significant differences between groups (Fig. 2).

For TA, a suspension effect on fiber axis was noted for type II fibers only (Fig. 3). Type I fibers' axis decreased in CON, HLS1W and HLS3D; however, significant differences were not found between each of them.

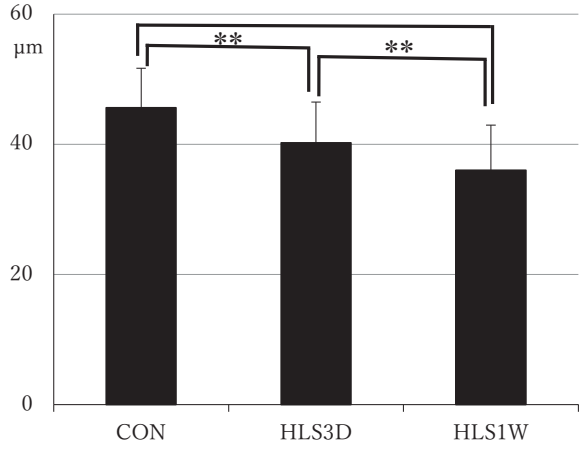

Figure 4 (**$p<0.01$)

In HLS3D and HLS 1 W, type II fibers in SOL had disappeared. Type I fibers' axis decreased in order of HLS1W, HLS3D, and CON. All comparisons between each other showed statistical significances (Fig. 4).

Discussion

Each HLS group exhibited statistically significant growth retardation. According to Chowdhury P et al.[2], HLS rats lost some weight during the first 3 days, which is consistent with results from the present study. Additionally, it is considered that HLS rats consume less than CON rats throughout the experiment[3], and as a result, diminished intake caused weight loss. Also, suspension had a less significant effect on bones than muscles; however, bone mineral density decreased significantly by 6% (Yamauchi et al.[4]). This decrease was thought to be one of the reasons for the body weights lost. As muscle wet weights are presented in Figure 1, EDL and TA mass resulted in a 25% and 20% loss respectively ($p<0.05$), compared to CON. In comparison, SOL muscle mass markedly decreased by 31%. This suggests that the atrophy is more severe in posterior muscles of leg as compared with anterior ones. When rats were suspended, their hindlimbs were presented with plantarflexion. This indicates that SOL muscle stayed in a shortened position while suspended. Preserving a shortened position for SOL resulted in significantly less muscle wet weights[5].

Similarly, maintaining a shortened position also had an effect on muscle fiber minor axis. In SOL, type

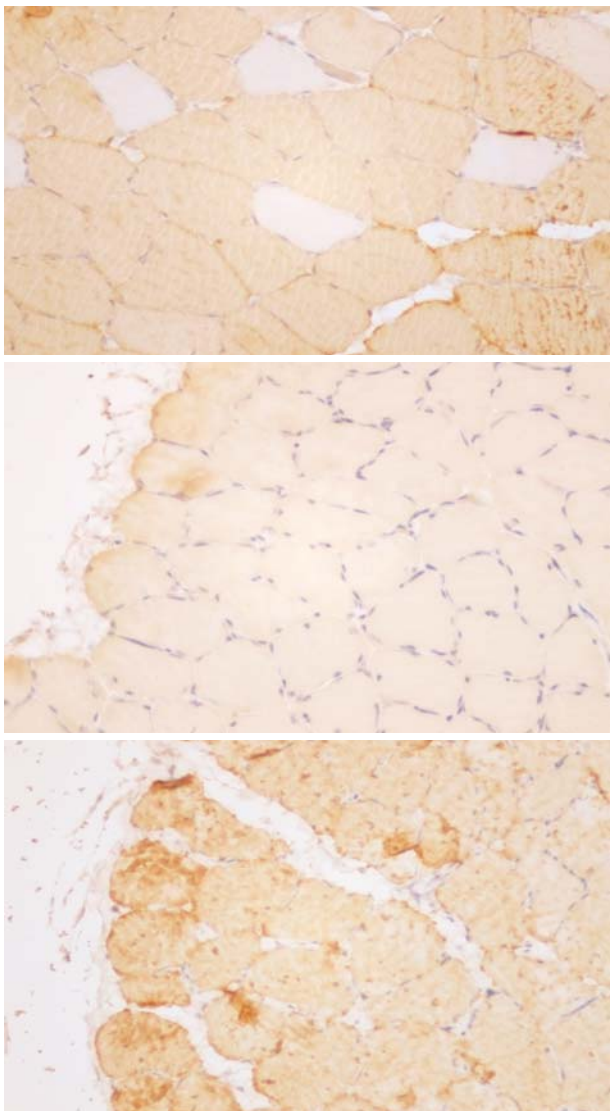

Figure 5 (×400)
Top: SOL CON
Middle: SOL HLS3D
Bottom: SOL HLS1W

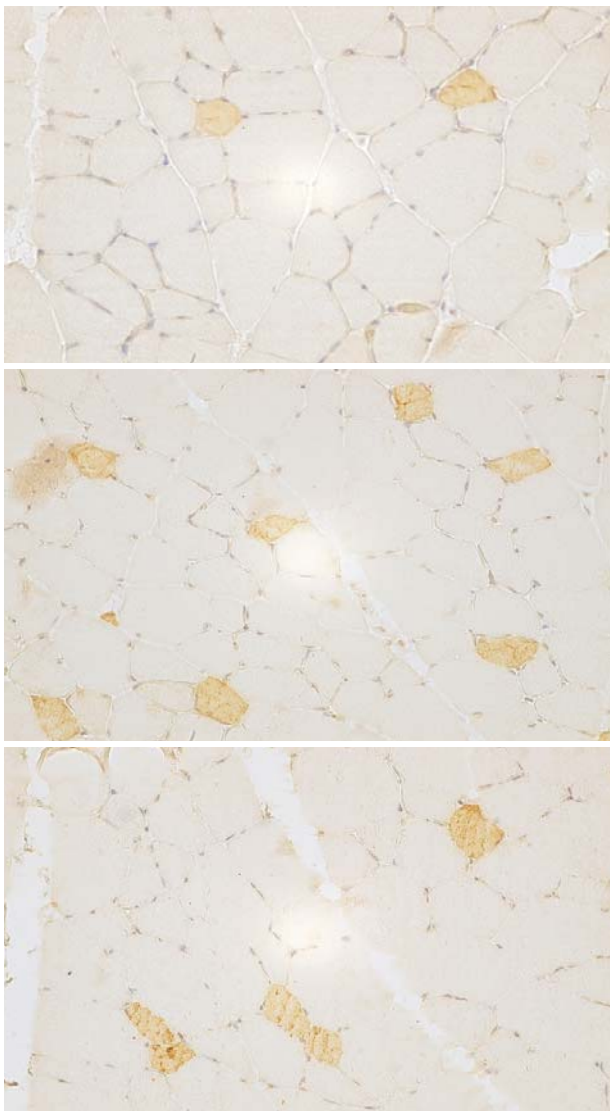

Figure 6 (×400)
Top: EDL CON
Middle: EDL HLS3D
Bottom: EDL HLS1W

I fiber's axis was associated with a statistically significant reduction in HLS3D and HLS1W, compared to CON. However, no significant differences were observed in EDL and TA. That is because both of them were stretched in a lengthened position.

Control soleus muscles contained two types of MHC (Myosin Heavy Chain) isoforms--fast type-MHC IIa and slow type-MHC I (The MHC isoforms I, IIa, IIb and IId were represented by the histochemically identified fiber types I, IIA, IIB and IID, respectively).

Oishi Y et al.[6] observed multiple type II MHC isoforms, including types IId and IIb, expressed in soleus single fibers after hindlimb suspension, and found four types of MHC hybrid single fibers: I+IIa, IIa+IId, I+IIa +IId, IIa+IId+IIb. Single fibers containing only the MHC I decreased, and fibers containing only MHC IIa isoform disappeared after hindlimb suspension. In addition, based on the study by Talmadge et al.[7] , who suggested the MHC conversion in rat soleus muscle during the unloading state as I→IIa→IIx→IId, the following scheme for MHC conversion may be considered: IIa→IIa+IId→IIa+IId+IIb. The appearance of hybrid fibers made type IIa fibers undetected after hindlimb suspension (Fig. 5). McDonald[8] et al' s results showed a 10% higher hybrid content in control soleus, which increased to 18% after 1 week of hindlimb suspension. McDonald and colleagues considered increasing hybrid fibers as a transient phenome-

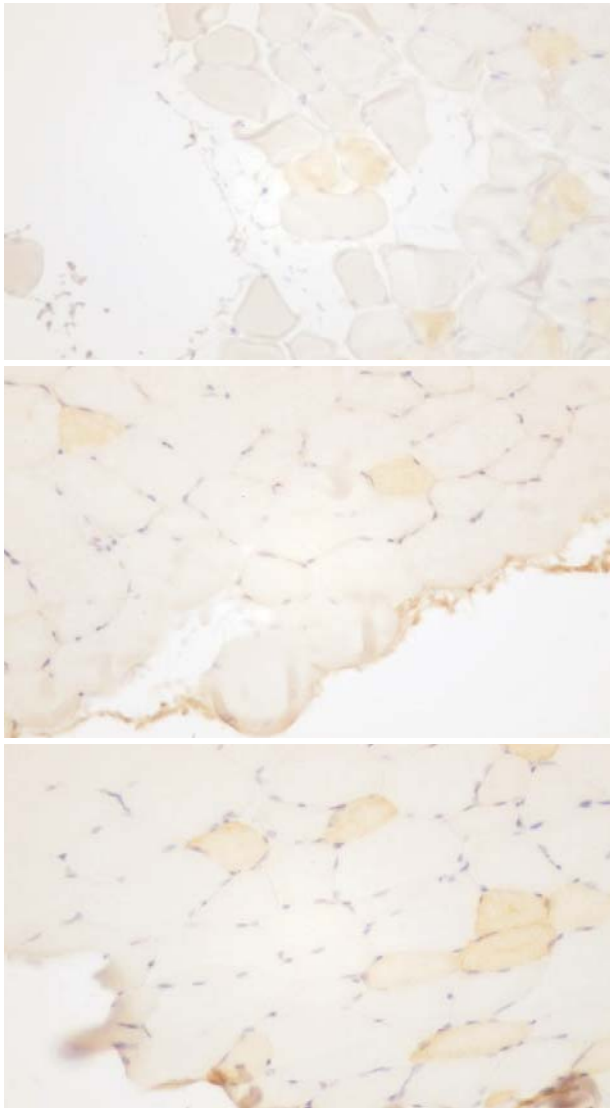

Figure 7 (×400)
Top: TA CON
Middle: TA HLS3D
Bottom: TA HLS1W

non and indicated that hindlimb suspension might induce the synthesis of a second, yet unidentified, slow MHC that co-migrates with slow type I myosin on 12% SDS gels. Given disappearing type II fibers in SOL, the present research suggested an induced synthesis of the unidentified slow MHC.

As for EDL in HLS3D, reloading fixed muscle atrophy and subsequently, there was no statistical significance between CON and HLS3D (Fig. 6).

In TA muscle, type II demonstrated hypertrophy throughout the week (Fig. 3). Goldspink[9] examined protein metabolism while holding a muscle in a lengthened or shortened position. As a result, EDL restrained in a shortened position showed decreases in protein synthesis, ribosomal availability, and ribosomal involvement in the translational process. In contrast, SOL in a lengthened position showed increases in protein synthesis, ribosomal capacity, and ribosomal efficiency, and hence induces the growth of muscles (Fig. 3, 7).

The muscle metabolism is built on dynamic balance between synthesis and degradation, and therefore, muscle tension has a significant effect on its regulation.

Conclusions

The disuse atrophy of type I muscle fibers occurred at an earlier stage than that of type II muscle fibers. It might lead to the decrease of walking ability because type I muscle fibers are mainly used for postural maintenance or endurance exercises. This suggests the importance of getting out of bed and starting an exercise program as soon as possible after surgery.

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