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SCIENTIFIC RESEARCH ARTICLE

Influence of Passive Stretching on Inhibition of Disuse Atrophy and Hemodynamics of Rat Soleus Muscle

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ABSTRACT. The purpose of this study was to determine the influence of passive stretching on inhibition of disuse atrophy and hemodynamics among longitudinal regions of the rat soleus muscle. Disuse muscle atrophy was induced by hindlimb suspension for two weeks. Muscle blood flow was evaluated using thallium-201 (^{201}Tl) which is a radiotracer that has been reported to be useful to assess blood perfusion in skeletal muscle. Thirty-nine male Wistar rats were divided randomly into 5 groups: control (C: n = 10), a group with hindlimb suspension (HS: n = 7), a group with hindlimb suspension and stretching (ST: n = 7), a group receiving only a single session of stretching after the hindlimb suspension period that was killed just after stretching (HSB: n = 7), and a group receiving only a single session of stretching hindlimb suspension and stretching period that was killed just after stretching (STB: n = 8). From the results of the cross-sectional area (CSA) and the capillary-to-fiber ratio (C/F), muscle atrophy and inhibition of atrophy were shown more in proximal than in distal regions of experimental groups. These results suggest that the alterations of the C/F and CSA were different among muscle regions in experimental groups. These differences may depend on the level of stretching. Moreover, alteration of blood flow resulting from alteration of the mechanical environment had little influence on muscle atrophy or inhibition of atrophy.

Key words: soleus muscle, disuse atrophy, blood flow, stretch

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Hindlimb unloading or immobilization results in disuse muscle atrophy[1)]. In addition, it has been reported that hindlimb unloading results in decrease in not only muscle mass but also the number of capillaries[2)]. Therefore, it is predicted that blood flow and metabolism in the muscle also decrease when a decrease in the number of capillaries occurs. For prevention of disuse muscle atrophy, weight-bearing or mobilization is carried out at the bedside. However, clinically, weight-bearing cannot be applied to patients with long-term bed rest or soon after an operation. In these cases, muscle stretching is effective in the inhibition of disuse muscle atrophy. In addition, it has been established that mechanical stimuli such as stretching, besides inhibiting muscle atrophy, increase the number of capillaries[3−5)]. In most previous studies, the alteration in blood flow with therapeutic interventions and disuse muscle atrophy was investigated on the basis of changes in the number of capillaries, and few studies have evaluated the changes in blood flow directly. Moreover, the relationship of the number of capillaries and blood flow is also uncertain. Therefore, the purpose of this study was to determine the effects of muscle stretching on disuse muscle atrophy in terms of muscle blood flow using thallium-201 (^{201}Tl). ^{201}Tl is a radiotracer that has been reported to be useful to assess blood perfusion in skeletal muscle[6,7)]. We also examined the relationship between blood flow and the numbers of capillaries in the proximal, middle, and distal regions of the muscle belly. Previous studies using animal experiments

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have shown that muscle stretching is effective in the inhibition of disuse muscle atrophy[8–13]. However, most of these used the middle belly for muscle analysis, and few considered differences among longitudinal regions. Nishikawa *et al*.[14] investigated the effects of intervention on the recovery process related to reloading after disuse atrophy of the rat soleus muscle with respect to sites and reported that there are differences in the responsiveness to hindlimb suspension and subsequent reloading among longitudinal regions of the rat soleus muscle; therefore, we assumed that there are differences among longitudinal regions with similar responses in muscle stretching. Fujino *et al*.[2] suggested that the decrease in capillaries in disuse atrophy muscle can affect muscle function, and that it is necessary to clarify the relationship between muscle cells and capillaries. However, few studies have examined the relationship between capillaries and muscle fibers. The purpose of this study was to determine the relationship of blood flow and muscle atrophy and its inhibition among longitudinal regions of the rat soleus muscle.

Materials and Methods

Animals and stretching protocol

All procedures for animal care and treatment were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals at Kanazawa University, and protocols were approved by the Committee on Animal Experimentation of Kanazawa University (AP-070867).

Thirty-nine male Wistar rats (age, 8 weeks; weight, 227±34 g) were randomly divided into 5 groups: 1) control group under standard breeding (C group: n = 10), 2) hindlimb suspended (HS) group (HS group: n = 7), 3) HS and stretched (ST) group (ST group: n = 7), 4) a group receiving only a single session of stretching after the HS period that was killed just after stretching (HSB group: n = 7), and 5) a group receiving only a single session of stretching HS and ST period that was killed just after stretching (STB group: n = 8). HSB and STB group were made to evaluate the blood flow occurred immediately after the stretching.

Hindlimb suspension was based on that described in a previous report. Soleus muscle atrophy was induced by HS for two weeks with the aid of a jacket. Weight-bearing was the only restriction on hindlimb activity, while the forelimb maintained contact with the floor, thus allowing the rats free access to feed and water.

The stretching method was based on that described in our previous report. In this report[15], we suggested that its inhibitory effect on muscle atrophy may be dependent on the degree of sarcomere stretching that was induced in response to dorsal flexion of the ankle joint. In our previous study, rats were subjected to muscle stretching by having their ankle joints dorsally flexed by the load of their body weight. The load was set as 50% of body weight since, in preliminary experiments in this study, ankle dorsiflexion range of motion with the loading of 50% of body weight was equivalent to the weight load. To eliminate the effects of anesthesia, the C, HS, and HSB groups were also anesthetized. The ankle joints were dorsally flexed 60 times for 10 s with a 10 s interval between each 10 s period, 5 days a week for 2 weeks. After stretching, rats were returned to their cages and had their hindlimbs suspended.

Thallium measurement

Commercial ^{201}Tl was diluted in physiological saline solution to prepare the radioactive stock solution. Under diethyl ether anesthesia, 0.1 ml of the solution was injected intraperitoneally. Ten minutes after injection, HSB and STB groups underwent only a single session of stretching for 20 minutes. Thirty minutes after ^{201}Tl injection, the animals were sacrificed by excessive inhalation of diethyl ether for immediate tissue harvesting. Samples of blood and the soleus were removed in sequence. The tissues were weighed immediately.

The right soleus was subjected to spectrometry and autoradiography measurements. The radioactivity was measured by spectrometry using an Auto Well Gamma System (Aloka, ARC-500, Tokyo, Japan). The results were compared with a 1% injected dose standard to obtain the tissue uptake rate in terms of the radioactivity percentage of the injected dose per gram of wet tissue (%dose/g). The uptake corrections were performed on the basis of the blood ^{201}Tl retention rate. The muscles were embedded in Tissue Tec OCT Compound and frozen at −25°C. The frozen muscles were cut into cross sections at 50 μm thickness and dried at −25°C using a cryostat (Sakura Finetek, Tokyo, Japan). Imaging plates were exposed to the sections inside cassettes within a lead depository to avoid high background radiation. After a week of exposure, the imaging plates were read using a bio-imaging analyzer (Fuji, BAS 5000, MultiGauge). On the image, each sample was divided longitudinally into four parts (proximal: 0-25%, proximal-middle: 25-50%, distal-middle: 50-75%, distal: 75-100%) and measured for uptake distribution of ^{201}Tl.

Histochemical assessment

The left soleus of C, HS, and ST groups was used for histochemical assessment. It was thought that, in HSB and STB groups, the last session of stretching just before extraction has little effect on the number of capillaries and CSA. In addition, because these two groups in this study were those in which the evaluation of blood flow occurred immediately after the stretching, these two groups were excluded from the examination of CSA and C/F ratio. The muscles were embedded in Tissue Tec OCT Compound and frozen in isopentane pre-cooled in liquid nitrogen, and then stored at −80°C until use. The muscles were transversely divided into four portions of equal thickness, and were re-

Table 1. Cross-sectional area and capillary-to-fiber ratio of the soleus muscle

		C	HS	ST
Cross-sectional area (μm^2)	25% (proximal)	2285 ± 595	884 ± 362*	1168 ± 474*§
	50% (middle)	2372 ± 641	1001 ± 350*¶	1236 ± 454*§¶
	75% (distal)	2368 ± 591	1230 ± 503*¶†	1264 ± 537*§¶†
Capillary-to-fiber ratio	25% (proximal)	1.91 ± 0.41	1.23 ± 0.32*	1.64 ± 0.27
	50% (middle)	1.58 ± 0.30¶	1.40 ± 0.25	1.55 ± 0.33
	75% (distal)	1.91 ± 0.27†	1.46 ± 0.51*	1.53 ± 0.29*

Values are means±S.D. * $p < 0.05$ compared with C (control) group, § $p < 0.05$ compared with HS (HS for 2 weeks), ¶ $p < 0.05$ compared with the proximal (25%) region, † $p < 0.05$ compared with central (50%) region.

ferred to as the proximal (25%), middle (50%; two such regions), and distal (75%) regions in terms of the muscle length from the proximal fiber insertions. Each tissue sample was cut into 10-μm-thick sections in a cryostat (Sakura Finetek, Tokyo, Japan). Sections were then stained with hematoxylin and eosin (HE), and with alkaline phosphatase (AP), and observed and photographed under a microscope. The cross-sectional area (CSA) of muscle fiber was measured using image analysis software, Image J, targeting a minimum of 400 fibers randomly in each section and the mean CSA of each region of each group was calculated. The number of capillaries and muscle fibers was counted targeting a minimum of 300 fibers randomly in each section. The mean capillary-to-fiber ratio (C/F) of each region of each group was calculated.

Statistical analysis

All data are expressed as mean±SD (standard deviation). Values of $P < 0.05$ were considered statistically significant. We used two-way analysis of variance (ANOVA) to compare CSA, C/F ratio, and the tissue uptake distribution of ^{201}Tl, and then we performed one-way ANOVA. Tukey's test was conducted if the ANOVA indicated a significant difference.

Results

Table 1 show the fiber cross-sectional area in each region of soleus muscle and the capillary-to-fiber ratio (C/F). In the comparisons within the same group, there were significant differences between each region in the HS and ST groups. In these groups, CSA was larger in the order of proximal < center < distal region, and CSA of all regions in HS and ST groups were decreased significantly compared with those in the C group. CSA of the proximal, middle, and distal regions in the HS group decreased to 38%, 47%, and 52% of that of the C group, respectively. Similarly, CSA of the proximal, middle, and distal regions in the ST group decreased to 50%, 57%, and 54% of that of the C group, respectively. On the other hand, CSA of the proximal, middle, and distal regions in the ST group significantly increased to 132%, 123%, and 103% of that of the HS group.

In the comparisons within the same group, C/F ratios of the middle region were significantly lower than those of the proximal and distal regions. In the proximal region, C/F ratio of the HS group was decreased significantly compared with that in the C group, but differences between C and ST groups did not reach statistical significance. In the distal region, C/F ratios of HS and ST groups were decreased significantly compared with that of the C group.

Table 2 shows the results of measurements using an Autowell Gamma Counter and the results of autoradiography (ARG) measurements. ^{201}Tl uptake rates in C group were significantly higher than those in the other four groups and there was no significant difference among these four groups. In four groups except HSB, the distributions of ^{201}Tl uptake in the regions of 25-50% and 50-75% were significantly higher than those in the 0-25% and 75-100% regions. In addition, there were no significant differences between 25-50% and 50-75%, or between 0-25% and 75-100%.

In each region, there was no significant difference among five groups. In the ST and STB groups, the distribution of ^{201}Tl uptake in the 50-75% region was higher (not significant) than those in the HS and HSB groups. In addition, the distributions of ^{201}Tl uptake in the 25-75% region of ST and STB groups were approximately constant, similar to that in the C group. In the STB group, the peak of this uptake occurred in the 50-75% region. In the HS group, ^{201}Tl uptake distribution peaked in the 25-50% region, and tended to decrease over the distal region. However, in the HSB group, uptake in the 0-25% region increased, with a trend that was similar to that in the HS group.

Discussion

^{201}Tl is a radiotracer available commercially as thallous chloride (^{201}TlCl). In its monovalent form, it behaves like

Table 2. Uptake rates and distribution of ^{201}Tl in the soleus muscle

		C	HS	ST	HSB	STB
Uptake rates (%dose/g)		0.45 ± 0.09	0.18 ± 0.09*	0.15 ± 0.12*	0.13 ± 0.05*	0.12 ± 0.07*
Distributions (%)	0-25%	19.9 ± 5.9	19.7 ± 6.1	19.2 ± 2.7	24.9 ± 4.6	19.7 ± 3.4
	25-50%	31.6 ± 3.1§	33.8 ± 3.9§	32.7 ± 2.0§	31.3 ± 3.3	30.1 ± 5.2§
	50-75%	31.8 ± 5.4§	28.7 ± 6.7§	31.3 ± 1.4§	27.3 ± 5.1	32.1 ± 3.4§
	75-100%	16.7 ± 4.0¶†	17.8 ± 3.7¶†	16.8 ± 4.0¶†	16.5 ± 3.5¶†	18.1 ± 4.1¶†

Uptake rates are expressed as percentages of administered dose taken up per gram of wet tissue weight (%dose/g). Distributions are expressed as percentage of ^{201}Tl uptake of the region in total uptake of the muscle. Values are means±S.D. * $p < 0.05$ compared with C (control) group, § $p < 0.05$ compared with the 0-25%, ¶ $p < 0.05$ compared with the 25-50%, † $p < 0.05$ compared with the 50-75% region.

an alkali metal ion, similar to potassium, and therefore it enters cells by similar transport mechanisms through blood flow after injection. The rapid uptake of ^{201}Tl provides early initial visualization of high-activity organ areas. This property has wide clinical applications, including in myocardial visualization, leg perfusion, tumor uptake, and thyroid uptake. The most common use of ^{201}Tl is in cardiac imaging. It can be used to assess not only myocardial perfusion, but also myocyte cell membrane integrity. Then, ^{201}TlCl is used to evaluate muscle blood flow by its initial distribution. The results of previous study demonstrated that ^{201}Tl is a very sensitive tracer for evaluating blood perfusion differences between hindlimb muscles[6,7].

^{201}Tl uptake rate as an index of blood flow was not influenced by hindlimb unloading and stretching, and did not increase in groups that underwent only a single session of these activities just before extraction. It has already been reported that changes occur in the structure and function of blood vessels as a result of hindlimb unloading. Fujino *et al*.[2] reported that muscle atrophy by hindlimb unloading causes regressive morphological changes in the capillaries, which result in decrease of the capillary growth factor. Delp *et al*.[16] reported that hindlimb unloading of rats resulted in a diminished ability of skeletal muscle arterioles to constrict in vitro and elevate vascular resistance in vivo. In addition, it has been established that blood flow is considerably decreased when stretching is applied acutely to muscles[4]. Poole *et al*.[17] observed that acute stretching of the spinotrapezius muscle resulted in a decrease in capillary diameter and velocity of flow.

In this study, it is thought that morphological changes and diminished function of capillaries resulted in decline of the blood flow.

However, the results of the trend of ^{201}Tl distribution suggest that there is a possibility that hindlimb unloading and stretching affected blood flow distribution. Although there was no significant difference, the results of the trend of blood flow distribution showed a tendency for an increase in the proximal region in HS (HS and HSB) groups and through the middle to distal middle region in the stretching (ST and STB) groups.

To show that these trends are related to the number of capillaries, C/F ratio was investigated. C/F ratio of the proximal region in the HS group significantly decreased compared with that in the C group, but there was no significant difference compared with that in the ST group. In the middle muscle region of experimental groups, there was no significant difference compared with that in the C group. In contrast, C/F ratio of the distal region in experimental groups significantly decreased compared with that in the C group. From these results, it was suggested that the influence on the number of capillaries of hindlimb unloading or stretching differs among the muscle regions, and it is the largest in the proximal region.

Hindlimb unloading results in a decrease in the number of capillaries. Fujino *et al*.[2] reported that regressive morphological changes in disuse atrophy muscle capillaries were induced by decreases of vascular endothelial growth factor (VEGF) mRNA and VEGF protein. Stretching results in increase in the number of capillaries[3]. Rivilis *et al*.[5] reported that stretching leads to capillary growth by endothelial sprouting with increased MMP-2 expression. Egginton *et al*.[4] reported that stretching can act as a mechanical stimulus to capillary growth, acting either directly on the capillary abluminal surface or by upregulating ESAF (endothelial-cell-stimulating angiogenic factor) in the extracellular matrix. Thus, the mechanism of the increase and decrease of capillaries has been reported and, in this study, the number of capillaries was decreased by hindlimb unloading, and was increased by stretching, especially in the proximal region. This result suggests that the changes of capillary number that resulted from hindlimb unloading and stretching are different among the muscle regions, which is an important finding.

Moreover, the difference of capillary number among the regions may affect the blood flow distribution. In other

words, it was considered that decrease in the number of capillaries in the proximal region with hindlimb suspension resulted in inhibition of blood flow to the distal region; on the other hand, inhibition of decrease in the number of capillaries with stretching resulted in blood flow distribution normalization. However, the tendency of blood flow distribution did not accord with the number of blood capillaries in this study.

Egginton *et al*.[4] reported the increase in capillary supply that has already occurred normalized any decrement in blood flow arising from the initial stretching. It has also been reported that the capillary growth induced in cases of skeletal muscle hypertrophy by long-term passive stretching is not initiated by any increase in blood flow. Therefore, the structural alternations, such as in wall thickness and diameter, or reactivity of the vessels, may affect the change of the blood flow. Further studies on this were needed.

It has been reported that disuse muscle atrophy progresses rapidly and is reached at 2 weeks by hindlimb unloading, and that stretching inhibits disuse muscle atrophy[8–13,15]. The effect of inhibition of disuse muscle atrophy by stretching was investigated separately in different regions. As a result, muscle atrophy by HS was found to progress more in the proximal region than in the distal region in HS group. Furthermore, the effect of inhibition of disuse muscle atrophy by stretching was larger in the proximal region and smaller in the distal region.

These results indicate that soleus muscle reactivity to the mechanical environment differs among regions and the proximal region is the most influenced by this. It was previously reported that the reactivity of skeletal muscle to mechanical stimulation differs among longitudinal regions[18–20], and our results support these previous studies.

Nishikawa *et al*.[14] demonstrated that, in soleus muscle of hindlimb unloading rat, muscle atrophy progressed significantly in the proximal region compared with that in the distal region. This was similar to our result. They suggested that the difference of degree of muscular atrophy with hindlimb suspension in the longitudinal region may have resulted from differences of protein synthesis and the resolution between longitudinal regions. In addition, since the distal region is near the Achilles tendon, the function of protection mechanism to prevent a rapid change of organization weight peculiar to a tendon tissue was to act, the degree of disuse muscle atrophy was small.

Wang *et al*.[21] reported that, after 16 days of hindlimb unloading, the number of satellite cells, which play an important role in the regulation of muscle fiber properties and in the recovery of CSA, was different among longitudinal regions, and the satellite cell-related regulation of muscle fiber properties is dependent on the level of mechanical loading, which, in turn, is influenced by the mean sarcomere length; sarcomere length is larger in proximal and distal regions than in the middle region.

For these previous studies, in our study, the difference of progression in disuse atrophy among regions was resulted from that the difference of level of sarcomere stretching among regions influenced in the protein composition or resolution, number of satellite cell and its activation.

In terms of CSA and C/F ratio, disuse atrophy was inhibited the most in the proximal region. From the report of Wang *et al*.[21], if the sarcomere in the proximal region is stretched, it is possible that the increase in the number of capillaries in the region is also influenced by the degree of stretching. Egginton *et al*.[4] reported that stretching can act as a mechanical stimulus to capillary growth, acting directly on the capillary abluminal surface. That is, it was thought that the fact that sarcomere length of the proximal region was larger by the stretching method of this study caused the reductions in the number of capillaries and muscle atrophy to be inhibited most in the proximal muscle part.

Moreover, from the results of the blood flow volume in these research findings, blood flow distribution and a CSA shows that there is little influence of the blood flow volume or blood flow distribution to reduction of CSA resulted from HS or inhibition the muscle atrophy by stretching.

Although no difference of blood flow volume was shown between HS and ST groups, specifically, the value of CSA was significantly higher in the ST group. In addition, the tendencies of blood flow distribution and CSA between the different longitudinal regions did not match. From these results, alteration of blood flow resulting from alteration in the mechanical environment had little influence on the muscle atrophy or inhibition of atrophy.

On of the capillary in disuse muscle atrophy with their functional alteration in hemodynamics is involving closely with muscle function. Although we did not evaluate muscle function in this study, it was suggested that blood flow distribution has a small influence on the morphological alteration of muscle.

Conclusion

In conclusion, the alterations of the number of capillaries and CSA differed among regions in the experimental groups. These differences may depend on the level of stretching in each region. Therefore, the inhibition of muscle atrophy may be more effective using the stretching method. In addition, it was indicated the possibility that blood flow distribution was affected by stretching. These results suggest that stretching may be effective for not only morphological aspects but also functional aspects of muscle. These are useful basic physiotherapy data, but further studies on the factors related to hemodynamic alteration and the influences of HS and stretching on myofunction are needed.

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