

Original Article

# Effects of static interventions on disuse atrophy of the rat soleus muscle at different sites along its longitudinal axis

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**Abstract.** [Purpose] The purpose of our study was to verify the inhibitory effects of static intervention (heat load and muscle stretching) on disuse-related adaptation changes in the soleus muscle and to compare these effects across different sites along its longitudinal axis. [Subjects] Forty 8-week-old male Wistar rats. [Methods] The effects of heat load and/or muscle stretching in the rat soleus during hindlimb suspension were evaluated by measuring the cross-sectional area of the muscle fibers, succinate dehydrogenase activity, and number of capillaries in the proximal, middle, and distal regions. [Results] With no intervention the proximal region showed the highest reduction in the cross-sectional area, whereas the distal region showed the highest reduction in succinate dehydrogenase activity and the number of capillaries due to hindlimb suspension. These differences between the proximal and distal regions decreased with both interventions, and the effects were most pronounced with a combination of heat load and muscle stretching. [Conclusion] Differences in the muscle structure between the proximal and distal regions increased due to hindlimb suspension, and this heterogeneity associated with muscle disuse was inhibited by static intervention including heat load and muscle stretching. Furthermore, the combination of heat load and muscle stretching most reduced the heterogeneity.

**Key words:** Heat load, Muscle stretching, Longitudinal axis

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## INTRODUCTION

Both the structure and function of skeletal muscle are adaptable, and it is well known that muscle disuse is accompanied by a decrease in the cross-sectional area (CSA) of the muscle fiber due to atrophy. Moreover, it is reported that muscle disuse decreases the mitochondrial volume, succinate dehydrogenase (SDH) activity<sup>1)</sup> and number of capillaries<sup>2)</sup>. This reduces aspects of myofunction that are important for activities of daily living, such as muscle contraction power and muscle endurance, which may lead to a further decrease in activity. Therefore, physical therapy intervention is important to prevent these changes.

Static interventions, which include heat load and muscle stretching without muscle contraction, have been reported to have an inhibitory effect on the progression of muscle atrophy<sup>3, 4)</sup>. In a previous study, peroxisome proliferator-activated receptor- $\gamma$  coactivator (PGC)-1 $\alpha$ , which facilitates

mitochondrial biogenesis and angiogenesis<sup>5)</sup> was activated by a heat load applied to a muscle cell<sup>6)</sup>. Therefore, a heat load is also considered to have an effect on muscle endurance. Furthermore, muscle stretching not only improves joint mobility but also has an inhibitory effect on muscle atrophy<sup>7)</sup> and acts on angiogenesis<sup>8, 9)</sup>. Sakaguchi reported that the use of a heat load and treadmill training had an inhibitory effect on muscle atrophy<sup>10)</sup>. Therefore, the use of muscle stretching, which is a mechanical load, combined with a heat load, can be expected to have a synergistic effect on the inhibition of the progression of disuse adaptation changes in the muscle.

Few studies have examined the effectiveness of a heat load and muscle stretching on the inhibition of the progression of disuse muscle atrophy along the long axis of a muscle. The distribution of the muscle fiber types, number of capillaries<sup>11)</sup>, and SHD activity<sup>12)</sup> differs along the longitudinal direction, and location-dependent differences in muscle adaptation changes and muscle structure have been reported previously<sup>13)</sup>.

This study sought to ascertain the effectiveness of static interventions for alleviating muscle atrophy caused by disuse, adaptive changes in the mitochondrial volume, and changes in the number of capillaries at different sites along the longitudinal axis of the muscle.

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## SUBJECTS AND METHODS

Forty male 8-week-old Wistar rats (mean±SEM body weight, 245.3±12.8 g) were included in the study. They were randomly assigned to one of the following five groups: control (CON, n=8); hindlimb suspension (HS, n=8); HS with heat load (HSH, n=8); HS with stretching (HSS, n=8); and HS with simultaneous heat load and stretching (HSHS, n=8). The breeding rooms were kept at a constant temperature (24±4 degrees centigrade) with free access to water and food and a 12-h light-dark cycle.

This protocol was approved by the committee for animal examination in the Kanazawa University (AP-122587).

The rats were suspended with the aid of a suspension jacket to prevent the hindlimbs from coming into contact with any supporting surface to prevent weight-bearing for one week.

A commercial heating pad (93×55 mm<sup>2</sup>, attakamonogari mini, Corporation Hakugen, Tokyo, Japan) was used to provide a heat load. The heating pad temperature was adjusted to approximately 39 degrees centigrade, wrapped in a paper towel, and attached to the lower limb for 60 minutes every day. Intraperitoneal anesthesia (pentobarbital, 50 mg/kg) was then administered. To control for side effects from anesthesia and the influence of pressure from the heating pad, an unheated heating pad was attached to the hindlimb in the CON and HS groups.

The stretching method was based on the description provided by Kimura and colleagues<sup>14)</sup>, in which a hand-made stretching device was used. During muscle stretching in a supine position, the hip and knee joints of the rats were in 90° flexion, and the ankle joint was stretched continuously in dorsiflexion for 60 min/day using a weight weighing 1/3 of the animal's body weight. During the stretching, an unheated heating pad was placed on the hindlimb of the HSS rats, and a heated heating pad was placed on the lower limb of the HSHS rats.

At the end of the 1-week experimental period, the animals' body weight was measured, and the right soleus muscle was removed under intraperitoneal anesthesia. After measuring the wet weight of the removed muscles, the muscles were rapidly frozen in isopentane cooled with liquid nitrogen. Then, the frozen samples were split into the proximal (first 25% from the origin of the muscle), middle (middle 50% of the muscle) and distal (furthest 25% of the muscle) parts. Each tissue sample was cut into 10 µm-thick sections in a cryostat (Tissue-tec Cryo3, Sakura Finetek, Tokyo, Japan). The sections were stained with hematoxylin and eosin (HE), SDH, and alkaline phosphatase (AP). For the SDH staining, the sections were incubated in sodium succinate, phosphate buffer, and nitro-blue tetrazolium for 30 min at 37 °C and fixed with acetone.

For the AP staining, the sections were incubated in borate buffer, α-naphthyl phosphate, fast blue RR, and magnesium sulfate for 60 min at 37 °C and fixed with formalin. Stain samples were observed and photographed under a microscope. The CSA and SDH activity of the muscle fibers and the number of capillaries around each muscle fiber were measured using the image analysis software Image J, targeting a minimum of 250 fibers randomly in each section. SDH

staining concentration was determined using constant light intensity. To evaluate SDH activity, the muscle fiber pixel values were subtracted from the background pixel value contrast.

The number of capillaries was determined to calculate the capillary-to-muscle fiber ratio (CFR).

We used one-way analysis of variance (ANOVA) to compare body weight, muscle wet weight, relative muscle weight ratio (muscle wet weight/body weight), and SDH activity of the muscle fibers. We used two-way ANOVA to compare the CSA and CFR across groups and muscle segments. Bonferroni's test was conducted if the ANOVA indicated a significant difference. A comparison of SDH activity among the groups at the same sites was not carried out. All data are expressed as the mean± standard deviation. P values <0.05 were considered statistically significant.

## RESULTS

The body weight and muscle wet weight were significantly higher in the CON group than the other groups, but no other significant differences in these variables were observed (Table 1). Additionally, there was no significant difference in the relative muscle weight ratio among the groups.

In the analysis of CSA, an interaction between site and group was found. At all sites, the CSA was significantly larger in the CON group than the groups treated with hindlimb suspension, and the lowest reduction of CSA values were observed in the HSH group (Table 2). Only the CSA at the proximal region of the HSHS group was significantly higher than the CSA at the same region in the HS and HSS groups. The CSA in the middle region was significantly higher than that in the proximal or distal regions in the CON group. However, the CSA was significantly decreased in the proximal region in all groups treated with hindlimb suspension. Only the CSA in the HSS group was not significantly different between the middle and distal regions, and in the HSHS group, there was no significant difference in CSA between the middle and proximal or distal sites. The ratio of the CSA at the proximal axis to the distal axis had the largest value in the CON group, followed by the HSHS group, and the HS group had the lowest value (Table 3).

A comparison of the SDH activity among the different sites in the same group (Table 4) revealed that in the CON group, and the SDH activity was significantly higher in the distal region whereas in the HS, it was significantly higher in the middle region and lowest in the distal region. SDH activity in the HSH and HSS groups showed a significantly lower value in the distal region than the middle or proximal regions. In the HSHS group, there was a significant difference between SDH activity in the middle region compared with the distal region. The ratio of the SDH activity at the proximal axis to the distal axis was the smallest in the CON group, followed by the HSHS, and highest in the HS group (Table 3).

The CFR displayed an interaction between site and group. In the HS and HSH groups, the middle and distal axes showed significantly lower CFR values than the CON group (Table 5). The CFR values in the middle region in the HS group and in the distal and middle regions in the HSH group

**Table 1.** Body weight, muscle wet weight, and relative muscle weight ratio of the soleus muscle

	Body weight (g)	Muscle wet weight (mg)	Relative muscle weight ratio (mg/g)
CON	253±11	93.6±8.1	0.37±0.03
HS	206±14*	68.4±5.2*	0.33±0.04
HSH	202±6*	67.6±11.0*	0.33±0.06
HSS	206±8*	67.1±5.0*	0.33±0.03
HSHS	200±13*	67.1±5.0*	0.34±0.03

All data are expressed as the mean±SD. CON: control, HS: hindlimb suspension, HSH: HS with heat load, HSS: HS with stretching, HSHS: HS with simultaneous heat load and stretching.

\*p <0.05 compared with the CON group.

**Table 3.** The ratio of the CSA, SDH activity, and CFR at the proximal to at the distal

	CSA	SDH activity	CFR
CON	0.995	0.720	1.002
HS	0.838	1.256	1.103
HSH	0.929	1.167	1.067
HSS	0.863	1.250	1.096
HSHS	0.955	1.040	1.048

All data are expressed as the mean. CSA: cross-sectional area, SDH: succinate dehydrogenase, CFR: capillary-to-fiber ratio, CON: control, HS: hindlimb suspension, HSH: HS with heat load, HSS: HS with stretching, HSHS: HS with simultaneous heat load and stretching.

**Table 5.** Capillary-to-fiber ratio of the soleus muscle

	Proximal	Middle	Distal
CON	1.82±0.43	1.82±0.34	1.81±0.39
HS	1.59±0.43	1.55±0.27*†	1.44±0.33*†
HSH	1.62±0.49	1.57±0.32*†	1.51±0.30*
HSS	1.75±0.22	1.70±0.26	1.60±0.30
HSHS	1.82±0.34	1.88±0.38	1.74±0.31

All data are expressed as the mean±SD. CON: control, HS: hindlimb suspension, HSH: HS with heat load, HSS: HS with stretching, HSHS: HS with simultaneous heat load and stretching.

\*p <0.05 compared with the CON group.

†p <0.05 compared with the HSHS group.

were significantly lower than the corresponding values in the HSHS group. There was no significant difference across the different sites in the same group. The ratio of the CFR at the proximal axis to the distal axis was smallest in the CON group, followed by the HSHS group, and highest in the HS group (Table 3).

## DISCUSSION

In this study, the ability of static intervention to inhibit the maladaptive changes associated with disuse in the skeletal muscle were compared across different sites along the

**Table 2.** Cross-sectional area ( $\mu\text{m}^2$ ) of the soleus muscle

	Proximal	Middle	Distal
CON	2,187±757	2,285±596§	2,199±596¶
HS	1,416±512*†‡	1,578±582*†§	1,689±542*†§¶
HSH	1,730±521*	1,785±620*§	1,863±479*§¶
HSS	1,412±400*†‡	1,660±568*†§	1,636±514*†§
HSHS	1,581±573*†	1,624±453*†	1,655±522*†§

All data are expressed as the mean±SD. CON: control, HS: hindlimb suspension, HSH: HS with heat load, HSS: HS with stretching, HSHS: HS with simultaneous heat load and stretching.

\*p <0.05 compared with the CON group.

†p <0.05 compared with the HSH group.

‡p <0.05 compared with the HSHS group.

§p <0.05 compared with the proximal region.

¶p <0.05 compared with the middle region.

**Table 4.** Succinate dehydrogenase activity (%) of the soleus muscle

	Proximal	Middle	Distal
CON	26.90±8.16	32.66±11.03*	37.35±11.51*†
HS	28.48±12.06	31.12±14.65*	22.68±11.36*†
HSH	30.80±10.22	29.05±9.74*	26.39±9.74*†
HSS	50.23±14.65	48.11±17.43*	40.19±14.69*†
HSHS	43.05±14.10	43.97±15.99*	41.78±18.69*†

All data are expressed as the mean±SD. CON: control, HS: hindlimb suspension, HSH: HS with heat load, HSS: HS with stretching, HSHS: HS with simultaneous heat load and stretching.

\*p <0.05 compared with the proximal region.

†p <0.05 compared with the middle region.

longitudinal axis of the muscle. Hedayatpour reported a non-uniform effect of exercise in the longitudinal direction that resulted in muscle strength imbalances, which may alter the load distribution on joint structures, thereby increasing the risk of injury<sup>13</sup>). Differences in the factors associated with disuse muscle atrophy across the sites on the longitudinal axis are also expected to cause muscle strength imbalances. It is therefore important to consider each site, and not only the entire muscle, when prescribing an intervention for muscle disuse.

In this study, there was a decrease in the CSA due to hindlimb suspension, and the addition of a heat load and muscle stretching affected the degree to which the CSA decreased. It has been reported that oxidative stress is reduced with increasing heat shock protein (HSP) 25 and HSP72 levels, thereby inhibiting the progression of muscle atrophy when a heat load is used<sup>4</sup>). Because the results of the present study were similar to those of previous studies, we consider the suppression of muscle atrophy to be the effect of increased expression of HSP triggered by the heat load. However, the muscle in the HSHS group, in which the heat load and muscle stretching were combined, exhibited a lower overall CSA than in the HSH group. This may be because in the HSHS condition, the muscle is expanded in the longitudinal direction by being stretched, thus the length

of the minor axis is possibly reduced. This CSA result is not inconsistent with the finding that there was no significant difference in the muscle wet weight. However, in the present study, we did not measure the muscle length, and this should be analyzed in future studies.

The number of capillaries was decreased in the middle and distal regions due to hindlimb suspension, and this decrease was inhibited by the combined use of heat load and muscle stretching.

Reports suggest that heat load has a pro-angiogenic effect on hindlimb ischemia secondary to the induction of endothelial nitric oxide synthase (eNOS)<sup>15)</sup> and HSP90<sup>16)</sup>. In addition, PGC-1 $\alpha$  is induced by the heat load on muscle cells<sup>6)</sup> it has also been implicated in angiogenesis with the generation of mitochondria during endurance exercises<sup>5)</sup>. Furthermore, Kanazashi has reported that the reduction in capillaries due to disuse is affected by increased oxidative stress. This is seen in rats treated with astaxanthin, in which antioxidant activity suppressed the increase in oxidative stress and the reduction of vascular endothelial growth factor (VEGF) and capillaries that was caused by hindlimb suspension<sup>2)</sup>.

In the present study, the increase in HSP by the heat load decreased VEGF and increased oxidative stress following disuse. As a result, there is a possibility that by combating the reduction in capillary angiogenesis, the heat load can mitigate the negative effects of disuse. Previously, it has been reported that capillary angiogenesis is promoted by the increase of VEGF and matrix metalloproteinases (MMP) due to muscle stretching<sup>17)</sup>. The results of this study suggest that the effect of angiogenic factors such as VEGF induced by HSP expression is enhanced when accompanied by a heat load and muscle stretching, which act synergistically.

Across the muscle longitudinal axis, the proximal site experienced greater atrophy caused by hindlimb suspension, but also experienced a greater effect of each intervention, than the distal axis. In contrast, SDH activity was decreased in the distal compared to the proximal region due to hindlimb suspension; additionally, the number of capillaries in the distal region also showed a tendency to decrease. The effect of each intervention on the SDH activity and number of capillaries was greater at the proximal site than distal site. Punk reported that the distribution of muscle fiber types in the soleus muscle is non-uniform in the longitudinal direction, and there is a higher number of proximal type I fibers<sup>18)</sup>. In this study, the greater atrophy in the proximal segment may have affected the muscle fiber type distribution. Difference in the expression level of HSP72 following a heat load are also affected by the distribution of type I and type II fibers<sup>19)</sup>, therefore heat load exerts a differential effect based on the muscle fiber type distribution along the longitudinal axis. De Ruiter et al. reported that the SDH activity is high in the proximal part of the medial gastrocnemius, and it has excellent fatigue resistance<sup>12)</sup>. Furthermore, they reported that the proximal and distal medial gastrocnemius have different contractile characteristics, as seen by electrically stimulating the nerve branches that supply the different sites of the medial gastrocnemius<sup>20)</sup>. In the present study, the differences in the changes caused by hindlimb suspension and the effects of the interventions are thought to be due to differences in the contractile characteristics of the longitudinal regions.

In addition, Mizuno reported a difference in blood flow distribution at rest (more blood flow in the proximal region) and during exercise (the difference between the proximal and distal regions was decreased) in the quadriceps muscle. This may be associated with the difference in the distance from the heart and the muscle fiber type distribution<sup>21)</sup>. It is considered that with the relative increase in blood flow at the distal sites induced by the heat load and muscle stretching, the reduction in the SDH activity and number of capillaries is inhibiting at the distal axis. Further, regarding the tissue structure, such as the muscle fiber type, it is conceivable that the difference in the stretching load is caused by the mechanical structure along the longitudinal axis, such as the flexibility and pennation angle of the muscle. However, in the previous study, consistent results could not be obtained regarding the pattern of site differences in morphology and adaptive changes along the longitudinal axis of the muscle based on animal type, target muscle, and differences in interventions. Factors such as muscle morphology, blood flow, and mechanical loads cause site differences, but the mechanistic details are unclear and require further study.

The most characteristic results of this study were the differences between the distal and proximal regions. The difference in the CSA between the proximal and distal segment was smallest in the control group, and increased in the following order: CON, HSHS, HSH, HSS, and HS. However, the relationship between the distal and proximal regions in terms of the SDH activity and number of capillaries was reversed, and while the differences were smaller, they occurred in the same order as the CSA. This result demonstrates an increase in the differences between the proximal and distal axes caused by disuse and suggests that the heat load possibly reduced the differences among the sites more than muscle stretching. It is presumed that a combination of heat load and muscle stretching will further reduce the heterogeneity along the muscle axial direction in a more effective manner.

In conclusion, differences in the muscle structure between the proximal and distal regions increased due to hindlimb suspension, and this heterogeneity associated with muscle disuse was reduced by static intervention including heat load and muscle stretching. Furthermore, a combination of heat load and muscle stretching reduced the heterogeneity to the greatest degree.

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