Effects of Static Load on the Weight and Protein Content in the Leg Muscles of the Mouse: a Simulation of Prolonged Standing in the Workplace

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Abstract: To simulate the effects of prolonged standing in the workplace on the leg muscles, we subjected mice to centrifugation for 6 wk. The absolute wet weight of leg muscles and internal organs of mice were measured after exposure to 3G by centrifugation for 6 wk and at 2 wk after removal of centrifugation. The weight of the soleus muscle (antigravity muscle) significantly increased after 6-wk exposure to centrifugation, but it decreased to its control weight 2 wk after removal of centrifugation. In contrast, the wet weights of the anterior tibial muscle, liver, and kidneys of mice centrifuged for 6 wk were significantly lower than those of the control mice; they had returned to control levels 2 wk after removal of centrifugation. It was therefore suggested that prolonged standing enlarged the leg muscles but its effect did not last for a long period of time after stopping prolonged standing. Western blot analysis of proteins extracted from the soleus muscle showed that vinculin and α-actinin in the centrifuged mice increased slightly, but there were no differences in the heat shock protein 70 (HSP70) and desmin levels between the centrifuged mice and control mice. No difference in HSP 70 suggested that muscle damage did not exist after 6 wk centrifugation.

Key words: Simulation of static load, Rat, Hypergravity, Soleus muscle

Introduction

Work requiring prolonged standing has been introduced in such occupations as assembly line work, quality-check work, shop clerk, nurse and flight attendant to increase work productivity. Prolonged standing was reported to cause health problems1–5) such as swelling and fatigue of the lower extremities, low-back or foot pain, etc. But it is not well known whether prolonged standing, which is a chronically static load on leg muscles, affects the muscle construction itself. To investigate the effects of prolonged standing to leg muscle (muscle weight, change in muscle protein content and muscle damage), we used an animal experiment. It is easy to control conditions and get leg muscle for study.

There were supposed to be several ways to load leg muscle: electrical stimulation, weight loading and hypergravity. In a chronic low-frequency electrical stimulation experiment6) which simulated chronic muscle use, it was reported that
some intermediate filaments, supposed to protect the ultrastructure of muscle fibers, increased in muscle. But the experiment on electrical stimulation had some problems: (1) Electrical stimulation has strong psychological effects on laboratory animals. (2) Laboratory animals jump in surprise on electrical stimulation. The load is not continuous and it does not simulate prolonged standing. The other way of loading was attaching heavy weight to the animal, but binding the weight to the body was stressful. Hypergravity could be considered a better method of loading the leg muscles. We did not have to stimulate directly or do any operations. Although such a stress as blood flow change and dullness of sense of balance were thought to be involved in centrifugation, they did not lead to a large amount of weight loss if the gravity was not so strong

As it was reported that in hypogravity environment the cytoskeletal protein content of leg muscle changed, protein content of leg muscle could be considered to change in a hypergravity environment. We expected that intermediate filaments which fortify the muscle ultrastructure would increase in muscle, since laboratory animals exposed to hypergravity needed more power to stand than with terrestrial gravity. Vinculin, α-actinin and desmin were among the intermediate filaments. Vinculin linked cells to each other and transmitted force generated in muscle to membrane. A previous study reported that a dynamic load created by chronic electrical stimulation of a muscle resulted in a significant increase in the level of vinculin. α-actinin also bound to cytoplasmic proteins and membrane receptors that regulate the structure of actin filaments. The interaction between α-actinin and other heavy proteins in the Z-disk suggested that α-actinin served to protect the structure of muscles. Desmin was believed to maintain the structural integrity of muscles because structural abnormalities of skeletal muscle were obvious in desmin knockout animals. Upon chronic stimulation of the anterior tibial muscle of rabbits, the desmin level increased with widening of the Z-disc as early as 2 wk after the onset of stimulation. Then we targeted these proteins to investigate if they would increase in hypergravity environment. It was also important to investigate whether the muscle was damaged by continuous static load. HSPs have strong cytoprotective effects and their levels increase when the organization of a muscle is damaged in an injurious environment such as high temperature, ischemia or exercise. The level of HSP70 in skeletal muscle increased depending on the exercise intensity. Then we targeted HSP70 among HSPs.

In the present study, mice were subjected to centrifugation for 6 wk to elucidate whether or not muscle weight and the relative amount of some protein in the leg muscle would change.

### Materials and Methods

**Animal care and hypergravity**

The study included 32 male ICR mice of 42 d of age. They were acclimatized for 7 d, and then placed into one of four groups: one of two stationary control groups (C1, C2) or one of two centrifuged groups (R1, R2). The mice in each group were housed 4 mice per cage (30.5 × 20 × 12.5 cm). The ambient temperature was 23°C with a light/dark cycle of 14 h/10 h. Ample food was put in a depression in the cage ceiling and drinking water was supplied from a bottle hung from the ceiling. The water did not drop during centrifugation. The mice in the centrifuged groups were continuously centrifuged at 3G for 6 wk on a rotor with a radius of 1.4 meters at 44 rpm. On the end of each arm of the rotor, a bucket which could swing smoothly was hung. In the centrifuge, mouse cages were put into buckets swung in order that the centrifugal force applied to the mice was vertical to the floor of the cages. The rotating speed kept constant by a feedback system was set at the beginning of rotation and observed at the end. The centrifuge was stopped for one hour every three days, during which food and drinking water were replenished and the body weights, amount of food and water consumed were measured. The mice in the control groups were placed under the rotor. After stopping the rotor and taking the mice out of the cages, we observed whether they could walk normally or not. Then each mouse was put on plate and its weight was measured with it on a balance while it did not move.

**Muscle preparation**

The mice in the R1 group were sacrificed immediately after the 6-week centrifugation, and the mice in the R2 group were sacrificed two weeks after the end of the six-week centrifugation. The mice in the C1 and C2 groups were sacrificed at the same time as those in the R1 and R2 groups. They were sacrificed by intraperitoneal injection of Nembutal® and the weights of the various organs were measured. The muscles and organs were dissected, cleaned of fat and connective tissue, weighed (wet weight), their tendons cut off, frozen in isopentane that was cooled by liquid N₂, and stored at ~80°C. The following muscles were excised from each leg: the soleus muscle (slow muscle), gastrocnemius muscle (fast muscle), anterior tibial muscle (fast), and extensor digitorum longus muscle (fast). The heart, liver, kidneys, lungs, spleen and diaphragm were also
dissected and weighed. Examinations of significant difference were done by Student’s t test for muscle weight, relative muscle weight and organs between R1 and C1, and between R2 and C2.

Muscle protein extraction

Proteins were extracted from the frozen muscles according to the method described by Chopard et al.8. Samples were pulverized in a small potter’s mortar that had been cooled by liquid N₂, and then homogenized in buffer containing 5 mM Tris, 10% Sodium Dodecylsulfate (SDS), 0.2 M 1,4-dithiothreitol, 1 mM Ethylenediaminetetraacetic acid (EDTA), and 2.5% protein inhibitor cocktail (Sigma, St. Louis, USA). Ten µl of buffer was added per 1 mg of muscle. The samples were boiled at 95°C for 1 min and then sonicated on ice. They were centrifuged (10 KG) at 4°C for 10 min.

Measurement of protein concentration

The total protein concentration in each sample was determined by means of a DC protein assay (Bio-rad, Hercules, USA). Bovine serum albumin (BSA) was used for the protein standard to calibrate the protein concentrations in each sample at a wavelength of 655 nm. The protein concentrations in the standards and samples were read three times and the mean values were used.

SDS gel electrophoresis

The samples were subjected to SDS- polyacrylamide gel electrophoresis (PAGE) according to the method of Laemmli17 at a constant current of 15 mA at 4°C for 80 min, with a 3–20% slab gradient SDS polyacrylamide gel (ATTO, Tokyo, Japan). We adjusted the samples for 10 µg protein/12 µl and poured 12 µl into each well. The gel was stained with Coomassie brilliant blue.

Western blotting

In the immunoblotting, a semidry electrophoresis transfer unit (ATTO, Tokyo, Japan) was used to transfer proteins from the gels to polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Bedford, USA), which were immersed in 100% Methanol (MeOH) for 30 sec and subsequently in H₂O prior to use, by the method of Kyhse-Andersen18 at 2 mA/cm² for 70 min. The PVDF membrane was washed in 0.15 M NaCl, 0.1 M Tris, pH 8.0, and 0.1% Tween 20 (TBST: Tris-Buffered Saline with Tween-20), blocked in 5% non-fat dry milk in TBST at room temperature for 1 h, cut into four pieces, and then hybridized with the monoclonal antibody (Sigma, St. Louis, USA) against vinculin (120 K), α-actinin (100 K), heat shock protein 70 (HSP70) (70 K), or desmin (53 K) at 4°C overnight. The dilution in TBST with 5% non-fat dry milk was 1:100 (anti-vinculin), 1:3000 (anti-α-actinin), 1:100 (anti-HSP70), or 1:3000 (anti-desmin). Blots were washed in TBST three times for 10 min each, incubated in anti-mouse IgG from sheep linked with horseradish peroxidase (Amersham Pharmacia Biotech, Piscataway, USA) diluted 1:5000 in TBST, and then washed in TBST three times for 10 min each. Chemiluminescence Reagent Plus (Perkinelmer Life Sciences, Boston, USA) was applied to the PVDF membranes and antibodies were detected by means of a digital image analyzer, LAX-1000Plus (Fujifilm, Tokyo, Japan).

Results

We observed the behavior of mice after stopping rotation. They walked awkwardly at the first measurement after three days’ centrifugation, but they could walk quickly from the next measurement. We observed the rotating speed at the beginning and end of the load, and they were the same.

Body weight

Although the initial body weights of the hypergravity and control mice were similar, the mice that had been placed under 3G centrifugation lost 9.8% of their body weight during the first 3 d of centrifugation, whereas the control mice gained 4.3% during the same interval (Fig. 1). At the third day of centrifugation, the mean weight of the centrifuged mice was 86.2% of that of the control mice, and food and water intakes were severely reduced. Nevertheless, the difference in the

Fig. 1. Weights of centrifuged and control mice after exposure to 3G.
On day 42, half of the mice were sacrificed and the number of mice in each group decreased from 16 to 8. After that, centrifuged and control groups were put in the same environment. A significant difference in weight was found between centrifuged and control mice until 40 d at p<0.01. But there was no significant difference in weight after cessation of centrifugation.
mean body weight between the centrifuged and control groups gradually decreased and the mean weight of the centrifuged mice was 95.0% of that of the control mice at six weeks of centrifugation. The food and water intakes of the centrifuged groups from the 3rd d to the 6th d of centrifugation recovered to about the same level as the control groups. The difference in weight decreased further to 98.0% two weeks after the cessation of centrifugation.

Muscle weight

The wet weight of various muscles changed differently after centrifugation for 6 wk, as shown in Table 1. The absolute wet weight of the soleus muscle of the centrifuged mice just after cessation of centrifugation was significantly higher by 32% than that of the corresponding control mice, but after two weeks the weight of the soleus muscle of the centrifuged mice decreased to that of control mice. On the other hand, the wet weight of the anterior tibial muscle of the centrifuged mice significantly decreased by 15% immediately after centrifugation for 6 wk, but after 2 wk the weight of the anterior tibial muscle in the centrifuged mice increased. The weights of the extensor digitorum longus muscle and gastrocnemius muscle did not show a significant difference after 6 weeks’ centrifugation and 2 weeks’ cessation of centrifugation.

Organ weight

The weights of the liver and kidneys were significantly smaller in the centrifuged group than in the control group immediately after cessation of centrifugation, but 2 wk after the removal of centrifugation, the significant difference between centrifuged and control mice disappeared (Table 2). The weights of the lung, spleen and diaphragm did not show a significant difference after 6 weeks’ centrifugation and 2 wk of no centrifugation.

Western blotting

As Western blotting was semi-quantitative method for protein, it was not suitable to express the proportion of protein numerically and analyze statistically, so we did not analyze the proportion of protein numerically and simply judged the protein increase or decrease by observation. The results

### Table 1. Effect of 3G centrifugation on muscle mass

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of mice</th>
<th>Soleus (mg) ± S.E.M.</th>
<th>EDL (mg) ± S.E.M.</th>
<th>Gastrocnemius (mg) ± S.E.M.</th>
<th>Tibialis anterior (mg) ± S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Just after six-week centrifugation (R1)</td>
<td>8</td>
<td>12.3 ± 2.1**</td>
<td>11.8 ± 1.3</td>
<td>177.6 ± 20.7</td>
<td>60.7 ± 5.8**</td>
</tr>
<tr>
<td>Control at six weeks (C1)</td>
<td>8</td>
<td>9.3 ± 1.2</td>
<td>12.1 ± 1.0</td>
<td>193.0 ± 12.5</td>
<td>71.3 ± 5.1</td>
</tr>
<tr>
<td>Two weeks after end of centrifugation (R2)</td>
<td>8</td>
<td>9.7 ± 0.7</td>
<td>11.8 ± 1.4</td>
<td>184.0 ± 12.2</td>
<td>65.4 ± 7.2</td>
</tr>
<tr>
<td>Control at eight weeks (C2)</td>
<td>8</td>
<td>9.2 ± 1.1</td>
<td>12.5 ± 1.2</td>
<td>196.9 ± 18.1</td>
<td>72.5 ± 5.9</td>
</tr>
</tbody>
</table>

Relative muscle weight (gms/100 g body weight) mean ± S.E.M.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of mice</th>
<th>Soleus</th>
<th>EDL</th>
<th>Gastrocnemius</th>
<th>Tibialis anterior</th>
</tr>
</thead>
<tbody>
<tr>
<td>Just after six-week centrifugation (R1)</td>
<td>8</td>
<td>3.3 ± 0.5**</td>
<td>3.2 ± 0.3</td>
<td>47.5 ± 3.8</td>
<td>16.4 ± 1.4*</td>
</tr>
<tr>
<td>Control at six weeks (C1)</td>
<td>8</td>
<td>2.4 ± 0.4</td>
<td>3.1 ± 0.2</td>
<td>49.7 ± 4.3</td>
<td>18.3 ± 1.3</td>
</tr>
<tr>
<td>Two weeks after end of centrifugation (R2)</td>
<td>8</td>
<td>2.4 ± 0.2</td>
<td>2.9 ± 0.3</td>
<td>45.4 ± 2.5</td>
<td>16.1 ± 1.6</td>
</tr>
<tr>
<td>Control at eight weeks (C2)</td>
<td>8</td>
<td>2.2 ± 0.1</td>
<td>3.0 ± 0.3</td>
<td>46.9 ± 4.7</td>
<td>17.3 ± 1.5</td>
</tr>
</tbody>
</table>

*P<0.05 Compared to the respective control, **P<0.01 Compared to the respective control, EDL; Extensor digitorum longus.

### Table 2. Effects of 3G hypergravity on mass of visceral organs

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of mice</th>
<th>Relative organs weight (gms/100 g body weight) mean ± S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Heart</td>
</tr>
<tr>
<td>just after centrifugation</td>
<td>8</td>
<td>0.411 ± 0.026**</td>
</tr>
<tr>
<td>control of above</td>
<td>8</td>
<td>0.460 ± 0.033</td>
</tr>
<tr>
<td>two weeks after centrifugation</td>
<td>8</td>
<td>0.403 ± 0.038</td>
</tr>
<tr>
<td>control of above</td>
<td>8</td>
<td>0.415 ± 0.035</td>
</tr>
</tbody>
</table>

*P<0.05 Compared to control, **P<0.01 Compared to control.
405A SIMULATION OF PROLONGED STANDING

of Western blot analysis of proteins extracted from the soleus muscle showed that there were small increases in the relative contents of vinculin and α-actinin in the centrifuged mice immediately after cessation of centrifugation in comparison with those in the control mice. The desmin expression was of the same level as the control. The level of HSP70, a candidate indicator of muscle damage, in the centrifuged mice immediately after 6-week exposure to 3G, did not apparently differ from that in the control mice (Fig. 2).

**Discussion**

Our experiment suggested that 3G hypergravity was an effective way to load leg muscle statically. The stress applied to the mice was not so great, because the weight increased normally. Exposure to 3G centrifugation for 6 wk increased the weight of the soleus muscle of mice and decreased the weight of the anterior tibial muscle significantly. It also increased the relative content of vinculin and α-actinin slightly, but two weeks after the return to terrestrial gravity, the weight of the two muscles did not significantly differ from those of the respective control mice.

During the first few days of exposure to centrifugation, the body weight of the mice decreased, when food intake was reduced. Reduced food intake would have a relationship to the decrease in body weight. A previous report also said that a decrease in food intake during the first few days of centrifugation would be one of the major causes of the sharp reduction in body weight. This would be supported by Pitts et al. who reported that a high-fat diet alleviated the suppressive effect of centrifugation on body weight.

After the first few days of exposure to centrifugation, the growth curve of the centrifuged group under centrifugation approached that of the control group and this suggests that the centrifuged group became accustomed to the stress of centrifugation. Studies on mice and white leghorns showed that during the first few days of centrifugation, the centrifuged group lost weight and, thereafter, the weight of the centrifuged group got closer to that of the control group. These findings suggested that long term centrifugation would not be a severe stress. As there was a little increase in weight after stopping the rotation, there could be other small stresses, such as dullness of sense of balance sense and change in blood flow.

The results of experiments on the effect of centrifugation on skeletal muscle mass have been controversial. Some studies on adult male rats reported that the absolute mass of the soleus muscle of adult male rats did not significantly differ from that of the controls, although the mass relative to the body weight increased in rats exposed to centrifugation. Other studies on mice and white leghorns reported that both the absolute and relative soleus muscle mass significantly increased in animals exposed to centrifugation. Another study on Wistar rats showed that the absolute mass of the anterior tibial muscle from centrifuged rats was significantly smaller than that from the control, although the muscle mass relative to the body weight of centrifuged rats was significantly greater than that of the controls. This inconsistency might have arisen from the difference in animal species used or their age, and the period or speed of centrifugation.

Theoretically, skeletal muscles that were subjected to centrifugation would become hypertrophic because they must sustain increased weight owing to the artificial changes in the ambient accelerative force caused by centrifugation. Electromyography (EMG) of the soleus muscle showed that centrifugation intensified the EMG activity of the soleus muscle and microgravity reduced it. The EMG activity of the anterior tibial muscle did not change upon exposure.
to centrifugation but it increased upon exposure to microgravity. These results supported our experiment that the weight of the soleus muscle increased but the anterior tibial muscle decreased under hypergravity. The anterior tibial muscle, a fast ankle flexor muscle that is used when walking forward, was thought to become thin because in the centrifuged environment the amount of exercise would diminish. Removal of centrifugation caused atrophy of the soleus muscle and hypertrophy of the anterior tibial muscle within two weeks. It could be suggested that the adaptation of the soleus muscle to prolonged standing might disappear in a short time and that the increased amount of exercise might increase the weight of the anterior tibial muscle.

We estimated, by observation, that vinculin, α-actinin and desmin would increase under hypergravity. Vinculin and α-actinin seemed to increase a little, which could indicate that these proteins helped to fortify the muscle structure. The desmin level did not seem to increase. Since desmin might increase in an earlier stage of centrifugation and decrease at 6 wk, a future study would be needed to measure the time course of the level of desmin for the soleus muscle of mice subjected to centrifugation.

Similarly we expected that HSP70 would increase in a hypergravity environment, because we expected that muscle would be damaged as the animals needed more power to stand and epidemiologically many workers had pain in the lower limb muscles during prolonged standing work\(^1\)\(^-\)\(^5\). Although six-week exposure to centrifugation increased the weight of the soleus muscle in this study, the HSP70 level in this muscle did not change. In our centrifuged mice, the exercise intensity may have been below the threshold of HSP70 production or the damage to the muscle may have recovered during the six-week centrifugation. Further studies on HSP70 production in the soleus muscle of mice that would be exposed to centrifugation with stronger hypergravity or for shorter periods would be needed.

To simulate prolonged standing, making the laboratory mice stand for a long time is supposed to be better, but regulating the behavior of mice would be difficult. Standing work requires the lower limb muscles to sustain the body at terrestrial gravity, though sitting work does not need force applied to the lower limbs. Then we loaded leg muscle with more hypergravity than the terrestrial one. To search for the obvious effects of load, mice were subjected to 3G, three times greater than terrestrial gravity. Although it had been estimated that mice might not stand up and move under hypergravity, the soleus muscle increased, which indicated that hypergravity was an effective way to load the soleus muscle.

It could be thought that hypergravity had caused other stress. But these stresses were not so large. One reason is that we used a bucket which swung out with rotating. Then the laboratory mice would feel that only the gravity increased. The other reason is that the centrifuged mice increased their weight at a higher rate than the control mice except for the first few days and walked normally just after cessation of rotation. Although other researchers\(^2\) reported that during hypergravity load labyrinthine sensitivity was reduced, the effect seemed to be small considering the overall conditions. The effects of microgravity on lower limb muscles are decreased considerably by returning the animal to terrestrial gravity for a few hours per day\(^2\)\(^6\). Therefore, in a future study mice should be subjected to centrifugation for periods corresponding to the number of working hours per day to simulate working conditions more practically.

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