Effects of Immobilization and Subsequent Low and High Frequency Treadmill Running on Rat Soleus Muscle and Ankle Joint Movement

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Abstract. To investigate the effects of low and high frequency treadmill running on immobilization-induced soleus muscle atrophy and ankle joint limitation, we performed morphological and histochemical analyses. Fifteen 8-week-old female Wistar rats (weight: 195.1 ± 6.2 g) were used in this study. After 2 weeks immobilization, rats were randomly assigned into 3 groups for 6 weeks exercise, such as free cage activity for the free remobilization (FR) group, once-a-week treadmill running (low frequency running program (LFR group)), six times a week running (high frequency running program (HFR group)). Two weeks of immobilization significantly reduced the soleus muscle wet weight, type I and II fiber cross-sectional areas, and range of ankle joint movement, and it increased the type II fiber ratios compared with the contralateral side. Some of these changes were not corrected by free remobilization, whereas in the LFR and HFR groups the changes were clearly restored toward normal levels, the effect being more beneficial in the HFR group for muscle recovery. In addition, LFR and HFR groups had improved range of ankle joint contracture in comparison with the FR group. These findings indicate that immobilization-induced muscle fiber histochemical alterations and the decrease of range of ankle motion in rats, to a great extent, is a reversible phenomena, especially if remobilization is intensified by physical exercise. High frequency running is more beneficial for recovery of the immobilization-induced muscle atrophy and joint contracture than no running or low frequency running.

Key words: Soleus muscle morphology, Joint contracture, Rehabilitation

INTRODUCTION

Skeletal muscle is a tissue that easily undergoes atrophy very quickly after cast immobilization, prolonged bed rest and denervation1–6). Among these conditions, immobilization is a frequently used treatment for musculoskeletal injuries. It is well known that immobilization induces muscle atrophy, intramuscular fibrosis, loss of muscle extensibility, and joint movement limitation1–6). Immobilization-induced muscle atrophy includes biochemical and morphofunctional modification such as atrophy of type I and II fibers7). These alterations include changes in fiber cross-sectional area, and fiber type composition5). Contracture of the joint by immobilization causes both muscle stiffness and changes in the connective tissue of the joint itself5).

Contrary to the large body of knowledge of immobilization, the effects of various forms of
remobilization procedure on alteration induced by immobilization are less well known, although the question is of utmost importance in rehabilitation medicine and sports medicine. Physical exercise is known to induce muscle hypertrophy and has been implicated in the modulation of muscle fiber behavior. In addition, exercise as a rehabilitation technique also helps to prevent muscle atrophy associated with disuse. Different physical therapies have been used to optimize muscle or joint functions and to treat muscle atrophy. Adequate rest periods are necessary to treat immobilization-induced muscle atrophy between bouts of exercise in order to facilitate the muscle recovery. However, the effects of a rest interval between training periods on change of atrophied muscle and joint contracture are less well known. Therefore, one of the aims of this study was to investigate the effect of once-a-week exercise on immobilization-induced morphological and histochemical alterations in rat soleus muscle and range of ankle joint movement.

In this study, we examined whether physical training would promote the rate of recovery after hindlimb immobilization and whether free cage activity, low or high frequency of exercise were effective for the recovery of immobilization-induced soleus muscle atrophy and joint contracture in the rats. Attention was also paid to the change of soleus muscle wet weight, fiber cross-sectional area (fiber size), fiber type composition and range of ankle joint movement in remobilization.

**MATERIALS AND METHODS**

*Animals and experimental protocols*

Fifteen 8-week-old female Wistar rats (weight: 195.1 ± 6.2 g) were used in this study. The following experimental protocol was approved by the ethical board of the Institute of Laboratory Animal Sciences of Kagoshima University. Three animals were housed per cage, and the animals received laboratory chow and water ad libitum. Using deep diethyl ether inhalation, three normal rats were sacrificed at the start of immobilization as non-immobilized controls. The remaining 12 animals were anesthetized with intraperitoneal pentobarbital (50 mg/kg) and the right hindlimb was immobilized in a plaster cast from the toes to about 1 cm above the knee. The knee was fixed in 90° flexion and the ankle in 70° plantar flexion so that the calf muscles were relaxed. The condition of the plaster cast in the hindlimbs of immobilized rats was checked every day. The plaster cast was repaired when necessary.

After 2 weeks of immobilization, three rats were sacrificed (the 2 week immobilization groups; IM group). In the remaining 9 animals, the plaster cast was removed and the animals were allowed to remobilize the right hindlimb for 6 weeks by using three different running protocols. The first three rats moved freely in the cage, and no additional physical training was given (the free cage remobilization group; FR group). The remaining two groups each with three rats run on a treadmill for 6 weeks. A motorized treadmill (Rat runner, RR-1200, AKK, Shimane, Japan) was used to perform a typical progressive resistance exercise. The program was progressive so that the running time increased from 10 min/session in the first week to 40 min/session in 6 weeks, and running speed was increased from 12 m/min to 24 m/min, with an inclination of 10°. In the group with a low frequency running program (LFR group), the treadmill running was performed once a week. In the group with a high frequency running program (HFR group), the treadmill running was 6 times a week. After the remobilization period for 6 weeks, FR, LFR and HFR groups were euthanized by deep diethyl ether inhalation.

In each animal, the range of movement of the ankle joint was measured every week under diethyl ether slight inhalation. In making the maximum plantar flexion to be the degree of zero, dorsiflexion of the ankle joint was measured to the resistance point using a protractor as described by Williams. In control animals, FR group, and each remobilization group after 6 weeks the range of ankle joint movement was measured on sacrifice.

In each animal, the hindlimbs were freed from the overlying skin and disarticulated at the hip. The soleus muscles of both legs were dissected out, cleaned of fat and connective tissues, weighed and quickly frozen in isopentane chilled with liquid nitrogen, and stored at −80°C for subsequent analysis. The samples for the microscopy of slow and fast twitch muscle fibers were taken from the middle part of the muscles of both limbs.

*Histologic and histochemical analysis*

Transverse serial sections, 10 µm thick, were cut with a cryostat microtome at −20°C and stained...
with hematoxylin and eosin (HE) for general observation. Sections were also stained for myosin adenosine triphosphatase (ATPase, pH 10.3, 4.3) reaction according to Guth and Samaha with some modifications. Sections stained for ATPase activity were used for classification of muscle fibers as either type I or II fibers.

Transverse sections from the muscle were used for morphometric study. The whole cross sections of each soleus muscle stained by ATPase were photographed at a magnification of 20× for fiber-type composition. Two regions of the muscle were photographed at a magnification of 50× at random so that the visual field did not overlap, and all the muscle fibers delineated by entire fiber boundaries were measured for cross-sectional area using computers and NIH Image software. A random sample of 200 fibers from each muscle was analyzed.

Data analysis

Data are expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) was used and when a significant F ratio was found, post-hoc Fisher’s protected least significant differences (PLSD) test was performed on each variable. Result were considered as statistically significant at values of P<0.05.

RESULT

Changes in the wet weight and the cross-sectional areas of each fiber type

The changes in the wet weights are shown in Table 1. There was no significant difference between the left and right sides of the soleus muscle wet weights of normal 8-week-old rats. Because individual muscle wet weight is related to the body weight, the muscle to body weight ratio was employed. Two weeks of immobilization created a significant decrease of wet weight (P<0.05). The ratios were significantly smaller than those of the contralateral control sides in FR and LFR groups (P<0.05). The wet weights were significantly increased in FR, LFR, and HFR groups compared with that of the IM group (P<0.05).

The changes in the muscle fiber cross-sectional areas are shown in Table 2. In contralateral nonimmobilized limbs of each group, the fiber cross-sectional area of the type I and II fibers was not significantly different compared with those of controls. Two weeks of immobilization created a significant decrease of both type I and II fiber cross-sectional areas (P<0.05). Both fiber types’ area significantly increased in FR, LFR and HFR groups (P<0.05). However, the area of both fiber types was lower than those of the contralateral side. There were no significant differences observed in type I fibers. 

### Table 1. The wet weight and the relative weight of the soleus muscle to the body

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>IM</th>
<th>FR</th>
<th>LFR</th>
<th>HFR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immobilized side</td>
<td>MW (mg)</td>
<td>53.0 ± 4.9</td>
<td>69.8 ± 6.4</td>
<td>70.5 ± 7.4</td>
<td>83.3 ± 5.3</td>
</tr>
<tr>
<td></td>
<td>MW/BW</td>
<td>0.27 ± 0.02</td>
<td>0.36 ± 0.02</td>
<td>0.37 ± 0.04</td>
<td>0.42 ± 0.03</td>
</tr>
<tr>
<td>Contralateral side</td>
<td>MW (mg)</td>
<td>80.3 ± 5.7</td>
<td>81.8 ± 6.9</td>
<td>84.8 ± 7.3</td>
<td>82.3 ± 4.3</td>
</tr>
<tr>
<td></td>
<td>MW/BW</td>
<td>0.40 ± 0.02</td>
<td>0.43 ± 0.04</td>
<td>0.43 ± 0.03</td>
<td>0.42 ± 0.04</td>
</tr>
</tbody>
</table>

Values are mean ± SD. IM: 2 week immobilization; FR: 2 week immobilization followed by free remobilization (free cage activity) for 6 weeks; LFR: low frequency exercise program, treadmill running was performed once a week; HFR: high frequency exercise program, treadmill running was performed 6 times a week. *: P<0.05 (compared with control). †: P<0.05 (compared with contralateral side). The control values derived from a total 6 soleus muscles: 3 left and 3 right sides.

### Table 2. The cross-sectional areas (µm²) of soleus muscle type I and II fibers

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>IM</th>
<th>FR</th>
<th>LFR</th>
<th>HFR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immobilized side</td>
<td>Type I</td>
<td>1539.7 ± 305.9</td>
<td>1998.1 ± 290.2</td>
<td>2008.1 ± 302.8</td>
<td>2583.6 ± 211.5</td>
</tr>
<tr>
<td></td>
<td>Type II</td>
<td>1104.9 ± 193.5</td>
<td>1543.8 ± 150.2</td>
<td>1608.1 ± 212.3</td>
<td>1927.2 ± 260.5</td>
</tr>
<tr>
<td>Contralateral side</td>
<td>Type I</td>
<td>2586.5 ± 305.9</td>
<td>2464.2 ± 265.7</td>
<td>2683.4 ± 280.9</td>
<td>2764.1 ± 301.2</td>
</tr>
<tr>
<td></td>
<td>Type II</td>
<td>1659.6 ± 289.6</td>
<td>1750.8 ± 248.2</td>
<td>1939.3 ± 190.8</td>
<td>1904.2 ± 191.2</td>
</tr>
</tbody>
</table>

Values are mean ± SD. *: P<0.05 (compared with control). †: P<0.05 (compared with contralateral side).
fibers between the FR and LFR groups. In addition, the area of type I fiber in the FR and LFR groups were significantly smaller than those of contralateral side. The area of type II fiber showed a similar change (P<0.05).

Changes in the ratio of type II to total fiber number

The changes in the ratio of type II fiber in the soleus muscle are listed in Table 3. The type II fiber ratio ranged from 5–10% in normal and contralateral soleus muscles. The ratios of type II to total fiber numbers in the IM group were significantly higher than those in the contralateral side and in the control (P<0.05). Many moderately stained fibers were recognized in the IM group, which were classified as type II fibers (Fig. 1). Type II fiber ratios were significantly decreased in FR, LFR and HFR groups (P<0.05). However, the ratio of the FR-group was significantly higher than that in the contralateral side and control (P<0.05).

Changes in the range of ankle joint movement

The changes in the range of motion of the rat ankle joints are shown in Table 4. The range of ankle joint of control and contralateral limbs were all 165 degrees. The range of ankle movement in the IM group was significantly reduced compared with control rats (P<0.05). The ranges of ankle movement significantly increased in the three remobilized groups (P<0.05). However, the range of ankle movement in three remobilized groups was significantly decreased until 3 weeks compared with control rats (P<0.05). The range of ankle movement in the FR group was significantly lower than in the two treadmill running groups (P<0.05). In addition, the ranges of ankle movement in the FR group had not recovered after 6 weeks (P<0.05).

DISCUSSION

Animal experiments studying the effects of remobilization on muscle structure and function

| Table 3. The ratio of type II fiber to total number after immobilization and remobilization |
|---------------------------------|-----------|-----------|-----------|-----------|
| Control                         | IM        | FR        | LFR       | HFR       |
| Immobilized side                | 25.6 ± 3.1* | 15.9 ± 5.8* | 9.5 ± 4.1  | 9.1 ± 7.1  |
| Contralateral side              | 7.1 ± 5.2   | 8.5 ± 6.1   | 5.5 ± 4.3  | 6.7 ± 7.3  | 5.7 ± 4.9  |

Values are mean ± SD. *: P<0.05 (compared with that of control value).

Fig. 1. Cross-section of the soleus muscle after 2 weeks of immobilization, stained by alkaline pretreated ATPase reaction. Bar=100 µm. A, contralateral soleus muscle, darkly stained type II fibers were limited in number; B, immobilized soleus muscle. Fiber cross-sectional areas were decreased in comparison with the contralateral side. Darkly stained type II fibers were significantly more numerous. Note that muscle fibers with various intermediate densities were classified as type II fibers in this study.
have been relatively few\textsuperscript{1, 4, 5, 12}. Kannus et al.\textsuperscript{5}) reported that immobilization-induced pathological structural and histochemical alterations in rat calf muscles were, to a great extent, a reversible phenomena if remobilization was intensified by physical training. Our results showed high frequency training facilitated the recovery of fiber size, ratio of type II fiber and range of ankle joint movement. These findings agree with those of Kannus et al.\textsuperscript{5}). However, in the present study, free remobilization increased the fiber size, but decreased the type II fiber ratio. This finding suggests recovery occurred naturally, even in the absence of intervention to facilitate recovery of atrophied tissues by physical training in the rat.

There were no significant differences observed in the muscle wet weight, type I fiber size and the ratio of type II fiber to total fiber numbers between the FR and LFR groups. The results suggest that low frequency treadmill running was not effective in facilitating recovery of atrophied tissues. Daily exercise given to rats increased fiber size of type I and II fibers\textsuperscript{5, 7}). In the present study, high frequency treadmill running significantly decreased the muscle wet weight, fiber size and the ratio of type II fiber to total fiber numbers. Our results suggest high frequency running is more beneficial for recovery of immobilization-induced muscle atrophy than low frequency running.

The soleus muscles were used because they are sensitive to immobilization atrophy and are known to respond well to physical training\textsuperscript{2, 4, 5}).

It is generally believed that a decrease in muscle activity facilitates a transformation of fiber type from slow to fast\textsuperscript{13}). There is evidence for specific degradation of the slow myosin isoform and possible enhanced synthesis of fast isoforms during hindlimb suspension that leads to an increase in relative amounts of fast myosin heavy chain\textsuperscript{14, 15}). The increase of type II fibers is observed not only in cast immobilization but also in denervation and spinal cord injury\textsuperscript{16–18}). During remobilization recovery, these changes seem to be reversible\textsuperscript{14, 15, 19}). Our result showed that type II fibers ratio of soleus was increased by immobilization and decreased by treadmill running. Fiber type transformation has been suggested as a mechanism responsible for endurance and strength training in animals\textsuperscript{12, 20}). It has been proved that a program of regular exercise training in both clinical and experimental assays can alter the distribution of different muscle fiber types\textsuperscript{5, 21}). The discrepancy in the extent of increase is perhaps, due to the increase in hybrid fibers containing both myosin heavy chains I and II at varying ratios within the same fiber\textsuperscript{17, 22}). This proposed conversion during immobilization and remobilization is still controversial.

Remobilization for 6 weeks restored the range of ankle movement to that of controls. Our result showed that the range of ankle movement of low and high frequency treadmill running was improved compared with those of free remobilization. This suggests that appropriate exercise after immobilization is more beneficial than free remobilization for range of movement and contracture.

CONCLUSION

Our study gives evidence that immobilization-induced reduction in fiber size, changes in fiber type conversion and joint contracture are, in the greater part, reversible phenomena. This study suggests that high frequency exercise is beneficial for recovery of the atrophied muscle tissues and joint contracture, but low frequency exercise has little effect. We demonstrated that high frequency exercise may help to facilitate recovery of muscle atrophy and joint contracture associated with immobilization.

ACKNOWLEDGMENTS

The author would like to thank Dr. Yoshihiro

\begin{table}
\centering
\caption{Range of ankle motion after immobilization and remobilization}
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
 & Control & IM & 1 week & 2 weeks & 3 weeks & 4 weeks & 5 weeks & 6 weeks \\
\hline
FR & 165 ± 0.0 & 74.4 ± 18.6* & 105.0 ± 10.0* & 107.0 ± 14.8* & 123.0 ± 21.6* & 119.0 ± 22.5* & 124.0 ± 21.9* & 122.0 ± 23.1* \\
LFR & 115.0 ± 12.6* & 126.7 ± 20.4* & 142.5 ± 14.1* & 139.2 ± 21.3* & 145.0 ± 12.2 & 147.5 ± 10.8 \\
HFR & 116.7 ± 10.3* & 135.8 ± 12.4* & 146.6 ± 7.5* & 155.8 ± 4.9 & 160.0 ± 5.4 & 160.0 ± 5.4 \\
\hline
\end{tabular}
\footnotesize{Values are mean ± SD. *: P<0.05 (compared with that of control value).}
\end{table}
Yoshida and Dr. Norio Morimoto for encouragement and advice.

REFERENCES