Effects of Walking and Weight-bearing Exercise on Soleus Muscle in Hindlimb-suspended Rat

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Abstract. [Purpose] This study examined the effects of walking exercise and weight-bearing exercise on the prevention of disuse muscle atrophy. [Subjects and Method] Male Wistar rats aged 7 weeks were used. They were randomly divided into control groups and experimental groups (n=8). After hindlimb-suspension for 7 days, the experimental groups were 1) hindlimb-suspended (HS), 2) exercised by treadmill walking (EX), 3) body weight-loaded (WB) and 4) re-loaded (RL) for 1 (day-8) or 7 (day-14) more days. The right soleus muscle was used for histology and stained with hematoxylin eosin and the left soleus muscle was used to measure the MyoD and mechano growth factor mRNA expressions. [Result] Soleus muscle wet weights and cross-sectional areas of RL-14 and WB-14 were significantly greater than those of HS-14, although those of EX-14 were not. Mechano growth factor mRNA expression of RL-8 was significantly higher than those of HS-8 and WB-8, but there were no significant differences between the values for RL-8 and EX-8. MyoD mRNA expression of RL-8 was significantly higher than those of the other groups. [Conclusion] Walking exercise couldn’t prevent disuse muscle atrophy any more than weight-bearing exercise, even though the mechanical stress of walking exercise was greater than that of the weight-bearing exercise.

Key words: Disuse muscle atrophy, Walking exercise, Mechano growth factor (MGF)

INTRODUCTION

Disuse muscle atrophy is caused by prolonged bed rest or the avoidance of weight-bearing, following restrictions of gait capacity and activities of daily living. Skeletal muscle is a highly plastic organ, the function and form of which are changed by the environmental situation. Thomason et al.1) reported that disuse muscle atrophy progresses rapidly and reaches a peak at 2 weeks. On the other hand, it has been shown that muscle hypertrophy is induced by some training and it is now possible to artificially induce hypertrophy by manipulating gene expression2). Satellite cells play an important role in muscle hypertrophy or regeneration because the muscle fiber itself does not have the capacity to divide3,4). Satellite cells are mononuclear cells located between the plasma membrane and the basal lamina in the adult muscle5). They are usually quiescent and are activated by various stimuli following initiation of the cell cycle and proliferation. Proliferated satellite cells fuse to produce new myotubes and/or fuse to existing myofibers, contributing to hyperplasia or increased numbers of muscle nuclei. In the course of this proliferation and differentiation, myogenic regulatory factors, for example, MyoD or myogenin, regulate muscle differentiation and activate the transcription of muscle-specific proteins6). MyoD is expressed when satellite cells proliferate and myogenin is expressed when satellite cells differentiate into myotubes.

It has been shown that insulin-like growth factor-1 (IGF-1) is secreted in an autocrine or paracrine manner when mechanical stress is loaded onto muscle cells7). There are two types of IGF-1, which are secreted by liver cells and by muscle cells, respectively. The latter IGF-1 is called mechano growth factor (MGF). MGF expression is increased by mechanical stress or myotrauma8,9). The increase of IGF contributes to an increase of myogenic regulatory factors, which are involved in the increase of muscle protein and the proliferation of satellite cells.

Weight-bearing10), stretching11) and electrical stimuli12) are effective for the prevention of disuse muscle atrophy. Weight-bearing is easily adapted to any clinical setting of physical therapy, and there are many reports on the effects of weight-bearing conditions, and stimulus time13) and frequency14). Brown et al.15) found that 1 hour of weight-bearing per day during 2 weeks of hindlimb-suspension prevented disuse muscle atrophy and suggested that weight-bearing was useful for the prevention of atrophy as a means of treatment. However, in the clinical setting, walking
SUBJECTS AND METHODS

Male Wistar rats aged 7 weeks were used in this study. All rats were given access to standard laboratory diet and water under a 12-h light/dark cycle. 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-091473).

Rats were randomly divided into control groups, 7 weeks (CON-1) and 9 weeks (CON-14), as well as experimental groups (n=8). After hindlimb-suspension for 7 days, the experimental groups were 1) hindlimb-suspended (HS-8, HS-14), 2) exercised by treadmill walking (EX-8, EX-14), 3) body weight-loaded (WB-8, WB-14) and 4) re-loaded (RL-8, RL-14) for 1 or 7 more days.

Hindlimb-suspension was based on that described in a previous report\textsuperscript{10}. Rats in the EX group performed running every day on a flat treadmill for a total of 60 min including rests. The exercise protocol, in accordance with the Armstrong protocol\textsuperscript{17}, involved intermittent exercise, in which the rats ran for 5-min exercise bouts at 0-10 m/min with 2 min rests. Rats in the WB group were housed normally for 60 min each day. After walking exercise and weight-bearing, rats were returned to their cages and hindlimb-suspended. Rats were injected with pentobarbital sodium (4.0 mg/100 g body weight), and intracardially exsanguinated. The right soleus muscles were extracted and quickly frozen in liquid nitrogen-cooled isopentane, then stored at –70°C until processing. The left soleus muscles were placed in stabilization reagent (Takara Bio, Shiga, Japan) with random hexamer primers. The reaction conditions followed standard protocols.

A quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used for analysis of mRNA expression. A Lightcycler\textsuperscript{6} (Roche Diagnostic Corporation, Tokyo, Japan) was used for qRT-PCR with SYBR\textsuperscript{®} Premix Ex Taq (Takara Bio) and primers (Table 1). Each primer pair was synthesized by Nihon Gene Research Laboratories (Miyagi, Japan). The PCR conditions of MyoD and glyceradehyde-3-phosphate dehydrogenase (GAPDH) were an initial step of 10 s at 95°C, followed by 5 s of denaturation at 95°C and 20 s of annealing and extension at 60°C. The condition for MGF was an initial step of 10 s at 95°C, followed by 5 s of denaturation at 95°C, 20 s of annealing at 57°C and 15 s of extension at 72°C. The number of cycles performed was 45 for MyoD and MGF, and 35 for GAPDH. The melting curve was produced by increasing the temperature at +0.1°C/s, and it was used to verify amplification product specificity. Relative expression levels of MyoD and MGF were normalized by subtracting the corresponding levels of GAPDH.

The results were analyzed using Smirnov-Grubbs analysis to exclude outliers (p<0.01). Mann-Whitney’s U test, Scheffe’s test or Steel’s test was then performed. All data for qRT-PCR are reported as mean values relative to CON-1. Values of p<0.05 were considered statistically significant. All data are shown as mean ± standard deviation (SD).

RESULTS

The results for body weight, muscle wet weight and CSA are shown in Table 2. Body weights of experimental groups on day-8 were significantly lower than that of CON-1. There was a significant difference between the body weights of CON-14 and RL-14, but that of RL-14 was significantly higher than that of the other experimental groups.

In terms of soleus muscle wet weight, those of HS-8, EX-8 and WB-8 were significantly lower than that of CON-1. Soleus muscle wet weight of RL-8 was significantly higher than that of HS-8, although those of EX-8 and WB-8 were not significantly higher. Soleus muscle wet weight of

### Table 1. Primers used for qRT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>MyoD</td>
<td>ACT ACA GCG GGC ACT CAG AC</td>
<td>ACT GTA GTC GGC GTC GT</td>
</tr>
<tr>
<td>MGF</td>
<td>GCT TGC TCA CCT TTA CCA GC</td>
<td>AAG TGT ACT TCC TTT CCT TCT C</td>
</tr>
<tr>
<td>GAPDH</td>
<td>AAC GGG AAA CCC ATC ACC A</td>
<td>CGG AGA TGA TGA CCC TTT TG</td>
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RL-8 was also significantly higher than those of EX-8 and WB-8. Furthermore, the soleus wet muscle weights of RL-14, and WB-14, were significantly higher than that of HS-14, and although the value for EX-14 was higher than that of HS-14, there was no significant difference. The soleus wet muscle weights of RL-14 was significantly higher than those of EX-14 and WB-14.

The staining with HE confirmed muscle atrophy in the HS group and recovery from atrophy was found in the RL group. We also found that the muscle areas of the EX and WB groups were larger than that of the HS group. The infiltration and accumulation of macrophages and the low density of cytoplasm were marked in EX-14 compared with the other groups.

In terms of CSA, those of HS-8 and RL-8 were significantly lower than that of CON-1. Only the value of EX-8 was significantly higher than that of HS-8; those of WB-8 and RL-8 were not higher. The CSA values of WB-14 and RL-14 were significantly higher than that of HS-14; there was no significant difference between the CSA values of EX-14 and HS-14. The value of CSA of RL-14 was also significantly higher than those of EX-14 and WB-14.

The results of qRT-PCR are shown in Table 3. MGF mRNA expression in HS-8 was significantly lower and that in RL-8 was significantly higher than that in CON-1. The expressions in RL-8 were significantly higher than those not only in HS-8 but also in WB-8. There was no difference between the expressions in RL-8 and EX-8. Although the expressions in RL-14 and EX-14 were relatively high, there were no significant differences among the experimental groups of day-14. MyoD mRNA expressions in WB-8, EX-8 and WB-8 were significantly lower than those in CON-1. The expressions in RL-8 were significantly higher than those of any other 8-day group. Although the expressions in EX-14 were higher than those of any other group, they were not statistically significant.

**DISCUSSION**

It has been reported the weight-bearing can prevent muscle disuse atrophy\(^{10,13,15,16}\). Although walking exercise is more easily adapted to clinical settings than weight-bearing exercise, there are few reports\(^{18,19}\) about walking exercise preventing disuse muscle atrophy. Therefore, this study was performed to examine the effects of walking exercise and weight-bearing exercise on disuse muscle atrophy using hindlimb-suspended rats. Rat’s hindlimbs were suspended for 1 week, following which they were exercised by treadmill walking or weight-bearing for 1 hour each day. The model of hindlimb suspension used in this study induced sufficient disuse muscle atrophy and the reloading stimulus prevented and enabled recovery from disuse muscle atrophy, as in past reports\(^{10}\).

In terms of soleus muscle wet weight, the weight-bearing for 1 hour prevented disuse muscle atrophy in a situation in which atrophy would otherwise have worsened, as previously reported\(^{15}\). Although soleus muscle weights in EX-8 and EX-14 were heavier than those in HS-8 and HS-
muscle atrophy\textsuperscript{11}) and low-load prolonged stretching was reported that prolonged stretching could prevent disuse muscle atrophy significantly, but this could not be determined from the results of this study because the period of hindlimb-suspension and the timing of intervention were different from those of the previous study. Although the duration of the weight-bearing exercise was as long as that of the walking exercise, interestingly, only the weight-bearing exercise prevented a decrease in soleus muscle weight. This result might have been influenced by not only the workload but also other factors. These other factors could include the influence of stretching. It was reported that prolonged stretching could prevent disuse muscle atrophy\textsuperscript{11}) and low-load prolonged stretching was more effective than high-load brief stretching\textsuperscript{20}). In WB groups, the soleus muscle was stretched for a longer duration than in EX, which was stretched intermittently, so the effect of prevention of disuse muscle atrophy might have been less in the EX group.

The CSA change in WB was prevented significantly compared with that in HS. Interestingly, the CSA change in EX-14 was not prevented since decrease of the soleus muscle weight in EX-14 could not be prevented. This result suggests that walking exercise might not always prevent disuse muscle atrophy. However, the CSA in EX-8 was significantly greater than that in any other group suggesting muscle fiber swelling rather than hypertrophy because the soleus muscle weight change in EX was not prevented and the CSA increase was only in the early phase after initial exercise.

It has been reported that reloading and exercise after hindlimb-suspension induce myotrauma\textsuperscript{8,21}). A micrograph of a sample from EX stained with HE shows that myotrauma occurred, confirming these previous findings. More interestingly, there was no significant difference between the CSA in HS-8 and RL-8. Thus, although walking or weight-bearing exercise for 1 hour induced muscle fiber swelling, reloading for 24 hours did not induce muscle fiber swelling. These results suggest that the degree of myotrauma is influenced by the speed of stretching\textsuperscript{23}) or the type of muscle contraction\textsuperscript{27}) rather than the extent of the muscle workload. In addition, there is a marked possibility that the stress of the intervention is also influential.

MGF, which is increased by mechanical stress, has a role in promoting cell proliferation. Heinemeier et al.\textsuperscript{23}) reported that MGF increased from 2 days to 8 days under reloading after 2 weeks of hindlimb-suspension. In our present study, MGF increased in RL-8. This increase was significantly higher than that in HS-8 and WB-8, suggesting that the weight-bearing exercise imposes great mechanical stress in disuse muscle atrophy and that the degree of mechanical stress is influenced by the amount of muscle work. Although MGF was not significantly increased in RL-14, in contrast to the findings of Heinemeier et al.\textsuperscript{23}), we think that our period of hindlimb suspension was shorter than in their study and that the initial condition of the muscle in each intervention was different. Thus, we consider the timing of intervention is important for preventing disuse muscle atrophy. The mechanical stress of the walking exercise was greater than that of the weight-bearing exercise because the MGF mRNA expression levels in the walking exercise were higher than those in the weight-bearing exercise, despite the two having the same period of exercise. It was mentioned that the WB groups may have experienced prolonged stretching compared with the EX groups, but it might be that the MGF mRNA expressions were dependent not on the stretching but rather the type of contraction, namely, isometric rather than isotonic.

It was reported that hindlimb suspension for 21 days made the MyoD, the label of satellite cell activation, expression increase\textsuperscript{24}). However, in this study, the levels of mRNA expression of MyoD significantly decreased, and this result might have been influenced by the duration of the hindlimb-suspension. On the other hand, as they increased in RL-8, many researchers have suggested that MyoD expressions are increased in regeneration after myotrauma\textsuperscript{25}). However, the degree of muscle fiber swelling in the EX group was greater than that in the RL group, and there was no increase of MyoD in the EX group. Therefore, the decrease of MyoD in the RL group was dependent on the amount of mechanical stress rather than the degree of myotrauma. There were no differences among any of the 14-day groups, but it was reported that the proliferation of satellite cells was observed at 30 h and the production of myotube was observed at 3 days after myotrauma\textsuperscript{26}). These results suggest that the satellite cells were activated and proliferated relatively early after initial exercise and that they proceeded to the next differentiation by 14 days.

The results of this study suggest that the growth factors, MGF and MyoD, have important roles in the prevention of disuse muscle atrophy. Walking exercise did not significantly prevent disuse muscle atrophy, and satellite cell activation was not induced by it. Interestingly, although the mechanical stress of walking exercise was greater than that of the weight-bearing exercise, it did not prevent disuse muscle atrophy. These results indicate that, in the clinical setting, forced walking exercise may not lead to significant prevention of disuse muscle atrophy and may induce muscle damage. The atrophied muscle is very fragile and has only a low exercise capacity, so it is very important in physical therapy for weak patients to think about phased programs.

REFERENCES


