Effects of Combination Therapy of Heat Stress and Muscle Contraction Exercise Induced by Neuromuscular Electrical Stimulation on Disuse Atrophy in the Rat Gastrocnemius

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Abstract. [Purpose] We investigated the effect of combination therapy, consisting of heat stress and muscle contraction exercise induced by neuromuscular electrical stimulation (NMES), for the prevention of hindlimb suspension (HS)-induced disuse atrophy in rat gastrocnemius (GAS) muscles, and clarified the effective exercise intensity for this therapy. [Methods] The experimental group was divided into the following six groups: 1) HS only; 2) HS plus heat stress (Heat); 3) HS plus low-intensity exercise (LEx); 4) HS plus combination therapy of heat stress and low-intensity exercise (H+LEx); 5) HS plus high-intensity exercise (HEx); and 6) HS plus combination therapy of heat stress and high-intensity exercise (H+HEx). Before, and at the end of the experimental period, muscle wet weight relative to total body weight, muscle fiber diameter, and heat shock protein (Hsp) 72 content in GAS muscles were evaluated. [Results] In the H+LEx and HEx groups, atrophy of all muscle fiber types in the deep and superficial regions was prevented. Hsp 72 expression was upregulated in the Heat, H+LEx, and H+HEx groups. [Conclusion] Our results suggest that low-intensity exercise is more effective than high-intensity exercise for the prevention of disuse muscle atrophy using heat stress and exercise combination therapy. The expression of Hsp72, which is induced by heat stress, may be related to this preventative mechanism.

Key words: Disuse muscle atrophy, Heat stress, Muscle contraction exercise

INTRODUCTION

Clinically, disuse muscle atrophy can occur in patients requiring prolonged periods of bed rest or immobilization of a joint in a plaster cast or orthosis after a fracture. Exercise is one of the most effective therapies for preventing muscle atrophy1–3). In general, exercising at ≤65% intensity for 1 repetition is not useful for gaining muscular size or strength4). This is a limiting factor for patients with various complications, such as internal tissue damage, mental disease and/or pain, as they are unable to undertake exercise ≥65% for 1 or more repetitions. Therefore, alternative treatments must be established for the prevention of muscle atrophy in these patients.

Recently, many studies have reported that heat stress improves disuse muscle atrophy, and it is believed that the heat shock protein (Hsp) 72 expression in skeletal muscle is related to this mechanism5–10). Hsp72 expression in cells is also stimulated by heat stress, depletion of glucose, and/or hypoxia11–17). Furthermore, Hsp72 has an important function as a molecular chaperone, whose expression may promote an increased rate of protein synthesis18, 19).

Studies using a rodent model of muscle disuse atrophy (i.e., hindlimb unweighting via tail suspension) indicate that the initial loss of muscle protein is primarily due to a decrease in the rate of protein synthesis20, 21). This decrease in protein synthesis is related to a reduction in the rate of nascent polypeptide chain elongation at the ribosomal level caused by a reduction in Hsp72 expression5–22). Additionally, oxidative stress is considered a potentially important regulator of proteolytic pathways leading to muscle atrophy during periods of immobilization9). Therefore, the elevation of cellular Hsp72 may serve as a countermeasure to attenuate disuse-induced muscle atrophy6–8). Kataoka et al. reported that the exposure of rat skeletal muscle to heat stress of 42 °C for 60 min per day, during tail suspension for one week, induced the expression of Hsp72 and prevented muscle atrophy in the soleus and extensor digitorum longus muscles23).
Decreases in muscle mass and strength occur in many patients undergoing rehabilitation; however, muscle atrophy is already advanced in many of these patients prior to the start of rehabilitation. In other words, remarkable muscle atrophy in these patients may be increased to the ratio of protein degradation. As previously stated, Hsp72 expression induced by heat stress has the potential to demonstrate the effect of the decrease of protein synthesis in the initial stage of progressive muscle atrophy. Therefore, we consider that the prevention of disuse muscle atrophy in patients is difficult using only single therapies, such as heat stress. Goto et al. compared the effects of low-intensity exercise (LIE) with and without heat stress on skeletal muscle of healthy individuals. In their experimental protocol, 60 min heat stress was applied to the non-dominant arm only, using a heat and steam-generating sheet, and LIE was performed by flexion and extension of the elbow joints, in both the non-dominant and dominant arms, from 30 to 60 min after the initiation of the heating procedure. LIE consisted of 3 sets of at least 30 repetitions, performed 4 days per week for 10 weeks. They found that LIE combined with heat stress induced an increase in the maximum isometric torque for 10 weeks. They found that LIE combined with heat stress induced an increase in the maximum isometric torque and cross-sectional area of the biceps brachii muscle.

Therefore, exercise combined with heat stress induced increases in muscle strength and hypertrophy. However, the optimal exercise intensity for the treatment of patients undergoing rehabilitation was not clarified. Additionally, the effect of exercise combined with heat stress for the treatment or prevention of disuse muscle atrophy has not been examined.

The aim of this study was to determine if the use of a combination therapy of heat stress and muscle contraction exercise by neuromuscular electrical stimulation (NMES) reduces the progression of disuse muscle atrophy in the rat gastrocnemius (GAS) muscle, and to determine the effective exercise intensity.

**MATERIALS AND METHODS**

Animal care and experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Nagasaki University and were approved by the Institutional Animal Care and Use Committee.

**Experiment 1 (Pilot study)**

In the pilot study, rat gastrocnemius (GAS) muscles were exposed to heat stress and the time course changes in Hsp72 expression were investigated. Based on the results, the frequency of heat stress was decided.

Thirty-five eight-week old male Wistar rats were randomly divided into two groups: control (Con, n=5) and heat stress (n=30) groups. Rats of the Con group were left untreated, whereas rats in the heat stress group were anesthetized and their bilateral hindlimbs were immersed in hot water of 42 °C for 60 min.

The right GAS muscles (five rats/time point) were removed at 4, 8, 12, 24, 48, or 72 h after heat stress treatment. In the GAS muscle, the superficial region comprises with fast-twitch fibers almost exclusively, but the deep region includes slow- and fast-twitch fibers. Therefore, after removing the muscles, they were immediately separated into two blocks of superficial and deep regions. Then, the muscle tissues were homogenized in 0.01 M phosphate buffer (PBS; pH 7.4) using a glass homogenizer. Homogenates were centrifuged at 4 °C at 12,000 g for 15 min, the supernatants were collected, and the protein concentration was measured using a BCA protein Assay Kit (Pierce Biotechnology Inc, USA). The final protein concentrations in the deep and superficial regions were adjusted to 1.5 µg/µl and 3.5 µg/µl, respectively. For SDS-PAGE and western blotting, Laemmli sample buffer (Bio-Rad Laboratories Inc, USA) and 2% 2-mercaptoethanol were added to each sample.

One-dimensional SDS polyacrylamide gel (12.5%) electrophoresis was performed to separate proteins by molecular weight. Samples, 15 µL, from each muscle were loaded onto the gels, and electrophoresis was performed at 20 mA (constant current/gel) for 90 min until the dye front migrated to the bottom of the gel. Following this, the gel was transferred to a polyvinylidene difluoride (PVDF) membrane using a mini trans-blot cell (ATTO Corp, JAPAN) at a constant voltage of 40 V for 70 min. After transfer, blots were blocked with 5% skim milk in Tris-buffered saline with Tween 20 (TTBS; 100 mM Tris-HCl, pH 7.5, 0.9% NaCl, and 0.1% Tween 20) for 1 h at room temperature. After washing five times in TTBS for 10 min each, the blots were incubated overnight with primary anti-Hsp72 antibodies (Stressgen Bioreagents Corp, CANADA) diluted 1:1000 in TTBS at 4 °C. After washing five times with TTBS for 10 min each, the blots were incubated with horseradish peroxidase-conjugated mouse anti-rat IgG (Medical and Biological Laboratories Corp Ltd, JAPAN) diluted 1:1000 in TTBS for 2 h at room temperature. The blots then were washed in TTBS and the membranes were subsequently reacted with a Metal Enhanced DAB Substrate Kit (Pierce Biotechnology Inc, USA). Quantification of the bands from each immunoblot was performed using a Scion Image System, and the data were expressed as a percentage of the content in the control group.

The data are expressed as mean ± SD. Overall differences were determined using one-way analysis of variance (ANOVA). When ANOVA results were significant, group differences were determined using Bonferroni’s post hoc test; p<0.05 was considered statistically significant.

Hsp72 content in the deep region was 259.4 ± 34.7, 317.8 ± 92.6, 181.9 ± 103.2, 241.9 ± 71.1, 158.4 ± 82.1, and 193.6 ± 108.4% at 4, 8, 12, 24, 48, and 72 h, respectively, after heat stress treatment. In the deep region, the Hsp72 content after 8 hours of heat stress was significantly increased compared with that of the Con group. The Hsp72 content in the superficial region was 498.0 ± 171.1, 689.6 ± 27.1, 492.7 ± 150.5, 570.4 ± 255.9, 413.1 ± 59.1, and 439.1 ± 202.4% at 4, 8, 12, 24, 48, and 72 h, respectively, after heat stress treatment. In the superficial region, the Hsp72 content after 4, 8, 12, 24, and 72 h of heat stress was significantly increased compared with the Con group.

These results suggest that the expression of Hsp72 in the superficial region is high until 72 h after heat stress, whereas
in the deep region it was only high for 8 h after heat stress. The application of heat stress once every three days (1 time in 72 hours) for the GAS muscle seemed likely to maintain high levels of Hsp72 expression. Therefore, we decided the frequency of heat stress for subsequent experiments to be once every three days.

Experiment 2

In this study, we examined the effect of using a combination therapy of heat stress and muscle contraction exercise induced by neuromuscular electrical stimulation (NMES) for the prevention of disuse muscle atrophy in the rat GAS muscle.

Thirty-six eight-week-old male Wistar rats were purchased from KYUDO Corp Ltd (JAPAN) and bred at the Laboratory Animal Center for Biomedical Research, Nagasaki University. All rats were housed in individual cages in a temperature-controlled room at 24 °C under a light-dark cycle of 12 h, and maintained on rat chow and water provided ad libitum. The rats were divided randomly into seven groups: 1) age-matched control (Con, n=5); 2) hindlimb suspension (HS, n=5) only; 3) HS plus heat stress (Heat, n=5); 4) HS plus low-intensity exercise by NMES (LEx, n=5); 5) HS plus combination therapy of heat stress and low-intensity exercise by NMES (H+LEx, n=5); 6) HS plus high-intensity exercise by NMES (HEx, n=6); and 7) HS plus combination therapy of heat stress and high-intensity exercise by NMES (H+HEx, n=5) groups.

Disuse muscle atrophy in the GAS muscle was induced in thirty-one rats via HS for 2 weeks similar to the method described by Naito et al. and Thomason et al. Briefly, the tail of the rat was wrapped with elastic tape and suspended by a wire attached to a swivel mounted at the top of the cage; this arrangement allowed each animal to perform 360° rotation. The height of each animal was adjusted to allow the rat to support its weight and move about freely on its forelimbs, and eat and drink freely while the hindlimbs were elevated to prevent contact with the floor.

Animals in the Heat, H+LEx, and H+HEx groups were anesthetized. Then, the bilateral hindlimbs were immersed in hot water using the same method of the pilot study, to apply heat stress. Based on the result of the pilot study, heat stress was performed once every three days 2 weeks of HS.

Muscle contraction exercise was conducted using NMES. NMES was adjusted to an intensity sufficient for regular muscle contraction. NMES was performed using an electro-stimulator (Torio 300, Ito Physio-therapy and Rehabilitation Ltd, JAPAN) and silver surface electrodes. The diameter of the electrodes was 10 mm, and they were placed on the inner and outer sides of the posterior surface of the hindlimbs of each rat. Animals in the LEx, H+LEx, HEx, and H+HEx groups were anesthetized, following which the bilateral GAS muscles were subjected to NMES (frequency, 50 Hz; pulse width, 250 μsec) for 20 min. The LEx and the H+LEx groups were given a stimulus intensity of 2 mA. At this intensity, muscle contraction was confirmed, but plantar flexion movement in the ankle joint enough was not induced. In contrast, a stimulus intensity of 4 mA was required to obtain plantar flexion movement in the ankle joint in the HEx and the H+HEx groups. We expected that it would be effective to use NMES when Hsp72 expression in the GAS muscle was high. Therefore, NEMS in the H+LEx and H+HEx groups was executed the day after heat stress treatment, and the frequency was once every three days during the 2 weeks of HS. Moreover, NEMS was performed at frequencies similar to those used for the LEx and HEx groups.

After 2 weeks of hindlimb-suspension, all rats were anesthetized with pentobarbital sodium (40 mg/kg) and bilateral GAS muscles were extracted and weighed. The left muscles were embedded in tragacanth gum, after which the samples were frozen in isopentane cooled by liquid nitrogen and stored at −80 °C. Serial frozen cross-sections of muscle, 7 μm in thickness, prepared on a cryostat, were mounted on glass slides for histochemical analysis. The right muscles were frozen quickly in isopentane cooled by liquid nitrogen, and stored at −80 °C until the relative content of Hsp72 was measured.

Some cross-sections of muscles were stained for myosin ATPase activity after acid pre-incubation (pH 4.2). The myosin ATPase reaction was examined to identify muscle fiber type. Muscle fiber diameter was determined for at least 200 fibers per major fiber type in the deep (type I, IIA and IIB) and superficial (type IIB) regions using image analysis software (Scion Image Software Program). Hsp72 content was measured using the same method as the pilot study.

The data are shown as mean ± SD. Overall differences were determined using one-way analysis of variance (ANOVA). When ANOVA results were significant, group differences were determined using Bonferroni’s post hoc test. Values of p<0.05 were considered statistically significant.

RESULTS

Table 1 summarizes the data for muscle wet weight relative to total body weight (MW/BW) and muscle fiber diameter in the deep and superficial regions of the all groups. The mean MW/BW of the H+LEx and HEx groups did not differ from the Con group. The mean MW/BW of the HS, Heat, LEx, and H+HEx groups decreased significantly in comparison with that of the Con group; however, the mean MM/BW of the H+LEx group increased significantly in comparison with that of the HS group (Table 1).

According to histochemical analysis, the deep region of the GAS muscle of all groups included type I, IIA, and IIB fibers; however, the superficial region of all groups were almost exclusively comprised of type IIB fibers. We divided the GAS muscles into deep and superficial regions and the mean muscle fiber diameters of each muscle fiber type in each group were compared. In the deep region, the mean diameter of type I muscle fibers of all experimental groups decreased significantly in comparison with the Con group, and the fiber diameter of type I muscle fibers of the LEx, H+LEx, HEx, and H+HEx groups increased significantly in comparison with the HS group. The mean diameter of type IIA and IIB muscle fibers of all experimental groups decreased significantly in comparison with those of the Con group, and the mean diameter of type IIA and IIB muscle...
fibrasts of the H+LEx and HEx groups increased significantly in comparison with those of the HS group. In the superficial region, the mean muscle fiber diameter of all experimental groups decreased significantly in comparison with the Con group, and the muscle fiber diameter of the H+LEx and HEx groups increased significantly in comparison with the HS group (Table 1).

Table 2 summarizes the data for the content of Hsp72 in the deep and superficial regions of all groups. In the deep region, Hsp72 expression was not different between the Con and HS groups; however, Hsp72 expression increased significantly in the Heat, H+LEx, and H+HEx groups compared with the Con group. In the superficial region, Hsp72 expression was not different between the Con and HS groups; however, Hsp72 expression increased significantly in the Heat group compared with that of the HS group. These results suggest that Hsp72 expression in the GAS muscle is upregulated by the application of heat stress once every three days for 2 weeks of HS; however, disuse muscle atrophy was not prevented. Previous studies have reported that around 1 week of HS-induced muscle atrophy is attenuated by heat stress (20, 21). Thus, the period of the HS may explain why the results of these reports differ from ours. Thomason et al. demonstrated that the loss of muscle protein after 3 days of HS was primarily due to a decrease in the rate of protein synthesis (20, 21). When HS was continued after this period, the rate of protein degradation increased for approximately 2 weeks, and the muscle atrophy became remarkable (20, 21). Therefore, the prevention of disuse muscle atrophy by heat stress may be effective during the period when decreases in muscle protein synthesis are high. As the initial decrease in protein synthesis is related to the reduction rate of nascent polypeptide chain elongation at the ribosomal level induced by the reduced expression of Hsp72 (18, 19), the elevation of cellular Hsp72 levels may serve as a countermeasure to attenuate disuse-induced muscle atrophy (6–9). Therefore, it is possible that muscle atrophy could be prevented through the induction of Hsp72 in muscle tissue by heat stress during the time period when muscle degradation is high. Thus, we hypothesized that muscle protein degradation in the GAS muscle would be very high after 2 weeks of HS. Our results suggest that it is difficult to control muscle protein degradation by the induction of Hsp72 expression using heat stress. Moreover, Goto et al. demonstrated that muscle hypertrophy due to heat stress occurs without the induction of Hsp72 expression in muscle tissue, and concluded that the frequency of the heat stress was an

**DISCUSSION**

In the HS group, the mean of MW/BW and muscle fiber diameters of all fiber types in both the deep and superficial regions decreased significantly compared with the Con group. These results indicate that disuse atrophy was induced in the GAS muscle of the HS group by hindlimb-suspension. Therefore, we assume that the methodology of HS used in this study is valid. The present findings demonstrate that Hsp72 expression in the GAS muscle did not decrease after 2 weeks of HS. Some previous studies have reported that muscle atrophy was induced after around 1 week of HS in adult rats, with decreased expression of Hsp72 in the soleus muscle (7, 8). In contrast, Desplanches et al. reported that Hsp72 expression in rat soleus muscles did not decrease after 2 weeks of HS, which is in agreement with our present findings (24). Their report noted that Hsp72 expression returned to control levels on the second week of HS because of a progressive increase in the rate of protein breakdown, and this change may be necessary to maintain the molecular chaperone function of Hsp72 by promoting its expression (24).

The mean values of MW/BW and muscle fiber diameter of all types of muscle fibers in both the deep and superficial regions in the Heat group did not differ significantly from those of the HS group; however, Hsp72 expression in the Heat group was significantly increased compared with that of the HS group. These results suggest that Hsp72 expression in the GAS muscle is upregulated by the application of heat stress once every three days for 2 weeks of HS; however, disuse muscle atrophy was not prevented. Previous studies have reported that around 1 week of HS-induced muscle atrophy is attenuated by heat stress (20, 21). Thus, the period of the HS may explain why the results of these reports differ from ours. Thomason et al. demonstrated that the loss of muscle protein after 3 days of HS was primarily due to a decrease in the rate of protein synthesis (20, 21). When HS was continued after this period, the rate of protein degradation increased for approximately 2 weeks, and the muscle atrophy became remarkable (20, 21). Therefore, the prevention of disuse muscle atrophy by heat stress may be effective during the period when decreases in muscle protein synthesis are high. As the initial decrease in protein synthesis is related to the reduction rate of nascent polypeptide chain elongation at the ribosomal level induced by the reduced expression of Hsp72 (18, 19), the elevation of cellular Hsp72 levels may serve as a countermeasure to attenuate disuse-induced muscle atrophy (6–9). Therefore, it is possible that muscle atrophy could be prevented through the induction of Hsp72 in muscle tissue by heat stress during the time period when muscle degradation is high. Thus, we hypothesized that muscle protein degradation in the GAS muscle would be very high after 2 weeks of HS. Our results suggest that it is difficult to control muscle protein degradation by the induction of Hsp72 expression using heat stress. Moreover, Goto et al. demonstrated that muscle hypertrophy due to heat stress occurs without the induction of Hsp72 expression in muscle tissue, and concluded that the frequency of the heat stress was an
important factor causing this change\(^9\). We hypothesize that it is not only Hsp72, but a number of factors that are related to the protective efficacy of heat stress against disuse muscle atrophy. However, this was not clarified in the present study and requires further study.

The mean values of MW/BW and muscle fiber diameter of types IIa and IIb fibers in the deep region and type IIb fibers in the superficial region in the LEx group did not differ significantly from the HS group. These results show that HS-induced muscle atrophy was not prevented by low-intensity exercise by NMES. In contrast, muscle fiber diameters of all fibers types in both the deep and superficial regions of the H+LEX group were significantly increased compared with those of the HS group, and Hsp72 expression was significantly increased in the H+LEX group compared with the HS group. Thus, these results suggest that the combination of heat stress and low-intensity exercise by NMES can attenuate the progress of disuse muscle atrophy. Goto et al. indicated that exercise training at an intensity ≤50% for a maximum of 1 repetition was effective for gain of muscular size and strength when heat stress was combined\(^{22}\).

Sakaguchi et al. demonstrated that the progress of disuse muscle atrophy in rat GAS muscles was not suppressed by treadmill running alone\(^{23}\). Additionally, their report showed that thermal loading prior to treadmill running inhibited the progress of muscle atrophy, and that thermal loading induced Hsp72 mRNA expression\(^{24}\). These results are in agreement with the results of the H+LEX group in our study. Moreover, Sakaguchi et al. noted that the combination therapy of thermal loading and treadmill running might be related to the upregulation of Hsp72 in muscle tissue\(^{25}\).

In the present study, the expression of Hsp72 in the H+LEX group was induced by heat stress. Therefore, we hypothesize that the protective efficacy of the combination of heat stress and low-intensity exercise for disuse muscle atrophy may be affected by the function of Hsp72 in myocytes. In our findings, the mean values of muscle fiber diameters of all fiber types in both the deep and superficial regions in the HEx group were significantly increased compared with those of the HS group. These results show that high-intensity exercise by NMES attenuates HS-induced muscle atrophy. In contrast, the mean values of MW/BW and muscle fiber diameters of type IIa and IIb fibers in the deep region and type IIb fibers in the superficial region in the H+HEx group did not differ significantly from those of the HS group. Furthermore, Hsp72 expression in the H+HEx group was significantly increased compared with that of HS group. Thus, our findings suggest the protective efficacy of the combination of heat stress and high-intensity exercise by NMES for muscle atrophy is not optimal despite the increased Hsp72 expression in myocytes. Moreover, we hypothesized that the beneficial effects of high-intensity exercise by NMES for muscle atrophy would be decreased by heat stress. Frier et al. demonstrated that Hsp72 expression was induced in myocytes following heat stress exposure to the rat hindlimb muscles, following which the plantaris muscle was exposed to mechanical overloading which caused muscle hypertrophy\(^{26}\). They also found that muscle hypertrophy in the plantaris muscle was not caused by the increase in Hsp72 expression, which is in agreement with the results of the H+HEx group in our study\(^{27}\). We also hypothesize that high intensity exercise does not easily induce muscle hypertrophy and strength when we use a combination therapy of heat stress and exercise, but were unable to confirm this in the present study. Conversely, Inami et al. reported that whole-body application of mild heat stress following a session of high-intensity training, which is associated with elevated blood lactate concentration, might enhance increases in muscle strength\(^{27}\). There is a possibility the combination therapy of heat stress and high-intensity exercise may differ when heat stress exposure is performed before or after exercise; however, this requires further study for clarification.

In our study, the effect of the combination therapy of heat stress and muscle contraction exercise induced by NMES for the prevention of disuse muscle atrophy in rat GAS muscles was examined, and the effective exercise intensity was clarified. In conclusion, our findings indicate that the combination of heat stress and low-intensity exercise by NMES is effective at attenuating disuse muscle atrophy, and this mechanism may be related to induction of Hsp72 expression in myocytes. As it is difficult for elderly patients and patients requiring bed rest to perform high-intensity exercise, the combination therapy of heat stress and low-intensity exercise may be an effective treatment option. Consequently, the data obtained in this study may help the development of a clinical application of this combination therapy.

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