Abstract

Electrical stimulation has been used to prevent muscle atrophy, but this method is different in many previous studies, appropriate stimulation protocol is still not decided. Although resistance exercise has also been shown to be an effective countermeasure on muscle atrophy, almost previous studies carried out an electrical stimulation without resistance. It was hypothesized that electrical stimulation without resistance is insufficient to contract skeletal muscle forcefully, and the combination of electrical stimulation and forceful resistance contraction is more effective than electrical stimulation without resistance to attenuate muscle atrophy. This study investigated the combined effects of electrical stimulation and resistance isometric contraction on muscle atrophy in the rat tibialis anterior muscle. The animals were divided into control, hindlimb unloading (HU), hindlimb unloading plus electrical stimulation (ES), and hindlimb unloading plus the combination of electrical stimulation and resistance isometric contraction (ES+IC). Electrical stimulation was applied to the tibialis anterior muscle percutaneously for total 573 sec per day. In the ES+IC group, the ankle joint was fixed to produce resistance isometric contraction during electrical stimulation. After 7 days, the cross-sectional areas of each muscle fiber type in the HU group decreased. Those were prevented in the ES+IC group rather than the ES group. The expression of heat shock protein 72 was enhanced in the ES and ES+IC groups. These results indicated that although electrical stimulation is effective to prevent muscle atrophy, the combination of electrical stimulation and isometric contraction have further effect. © 2011 Association of Basic Medical Sciences of FBiH. All rights reserved

KEY WORDS: muscle atrophy, electrical stimulation, resistance, isometric contraction, HSP72

INTRODUCTION

Skeletal muscle atrophy results from variety conditions, including unloading [1, 2], joint immobilization [3, 4], denervation [5-9], and spinal cord injury [10-12]. Electrical stimulation has been used to prevent muscle atrophy induced by these diverse conditions, and proved to be effective. Some studies have reported that electrical stimulation prevented decreases in the muscle mass [13, 14] and the muscle fiber cross-sectional area [9, 14], the myofibril disruption [5, 9], weakness of muscle contractile force [13, 14], transition from slow to fast myosin heavy chain [13], and the expression of muscle specific genes related to atrophic pathway [8, 12]. Whereas other studies have indicated that electrical stimulation could not prevent muscle atrophy [15, 16]. For the methods of electrical stimulation, there are differences among previous studies. Some studies have reported that the effect of electrical stimulation on muscle atrophy was influenced by stimulation intensity [6] and frequency [17, 18], chronaxie [8], number of contraction [6, 7], number of therapeutic session in a day [11], types of contraction [10], and electrode differences between surface [8, 9, 18] and implantation [5-7, 10, 13, 14, 17]. Therefore the effect of electrical stimulation depends on the methods, improvement of the methods is required to establish it as a more efficient countermeasure to muscle atrophy. Resistance exercise has also been shown to be an effective countermeasure on muscle atrophy [19, 20]. Some studies showed the preventive effect on muscle atrophy [21, 22] in response to resistance exercise. Although resistance exercise is regarded as one of the effective countermeasure, almost previous studies carried out an electrical stimulation without resistance. The electrical stimulation without resistance might be insufficient to contract skeletal muscle forcefully. We hypothesized that the combination of electrical stimulation and forceful resistance contraction is more effective than electrical stimulation without resistance to prevent muscle atrophy. For the prevention of skeletal muscle atrophy, it has been demonstrated that heat shock protein (HSP) 72 is associated...
with the attenuation of atrophy [2, 23]. HSP72 are known to contribute to reducing cellular damage, protect skeletal muscle against stressful events, such as thermal stress, ischemic injury, and oxidative free radicals [24, 25]. Electrical stimulation [26] and exercise resistance [27-29] have also shown to markedly increase in HSP72 expression, respectively. If overexpression of HSP72 is induced by the combination of electrical stimulation and forceful resistance contraction, it might have greater enhancement of the preventive effect on muscle atrophy. The purpose of the present study, therefore, was to assess the combined effect of electrical stimulation and forceful resistance contraction to prevent muscle atrophy. Furthermore, this study also investigated the level of HSP72 response to the combination of electrical stimulation and forceful resistance contraction. In the present study, forceful isometric contraction as a resistance exercise was produced by joint fixation during electrical stimulation.

**MATERIALS AND METHODS**

**Experimental groups**
The present study used 28 adult male Wistar rats (Japan SLC, Shizuoka, Japan) weighing in 242-265 g. The animals were randomly divided into four groups: 1) control (Cont), 2) hindlimb unloading (HU), 3) hindlimb unloading plus electrical stimulation (ES), and 4) hindlimb unloading plus the combination of electrical stimulation and forceful isometric contraction (ES+IC) groups. This study was approved by the Institution Animal Care and Use Committee and carried out according to the Kobe University Animal Experimentation Regulations. All experiments were conducted in accordance with the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals (National Research Council, 1996).

**Hindlimb unloading**
Hindlimb unloading was applied to animals by suspending their tails for seven days, according to the method described by Morey [30]. Briefly, each animal in the HU, ES, and ES+IC groups was fitted with a tail harness and was suspended by a string just high enough to prevent the hindlimbs from bearing weight on the floor or sides of the cage. The forelimbs were allowed to maintain contact with the floor of the cage and the animals had full access to food and water. The animals in each group were housed in an isolated and environmental controlled room at 22 ± 2°C with a 12:12-h light-dark cycle.

**Electrical stimulation protocol**
Electrical stimulation began the day after hindlimb unloading and continued for six consecutive days. The animals in the ES and ES+IC groups were anesthetized at the same frequency as in the ES and ES+IC groups to exclude the influence of anesthetic. Electrical stimulation equipment (SEN-3301, Ni-hon Kohden, Tokyo, Japan) that permitted changes in the electrical parameters was used to treat the tibialis anterior muscle percutaneously. Two surface electrodes were used to stimulate the tibialis anterior muscle. One electrode (5 cm in diameter) positioned on the animal's back, and the other active electrode (3 mm in diameter) was adhered on the motor point of the tibialis anterior muscle. During the electrical stimulation, the active electrode was maintained in contact with skin overlying the tibialis anterior muscle perpendicular to the muscle fibers. The stimulation was a positive square wave with pulse width of 0.1 ms, and the stimulation pulse amplitude was maintained at 4 mA. Each pulse was delivered at frequency of 100 Hz. During the electrical stimulation, pulses were delivered for 1 sec every 3 sec (time on: 1 sec; time off: 2 sec) for 1 min, followed by 5 min of rest. Six consecutive sessions of stimulation were performed twice in a day, separated by a 9-h interval. This resulted in a total stimulated duration of 240 sec in a day. In the ES+IC group, the ankle joint was fixed to produce a forceful isometric contraction at 90° in a removable plaster cast during the electrical stimulation (Figure 1). The force transducer (RX-1, Aikoh Engineering, Osaka, Japan) was attached in the dorsum of the foot to measure the torque at dorsiflexion of the ankle joint produced by electrical stimulation. The muscle contractile force evoked by electrical stimulation in the ES+IC group was approximately three times larger than that in the ES group (ES: 468 ± 66, ES+IC: 1216 ± 76 mN). In the rest time between sessions, the limb was removed from the cast.

**FIGURE 1.** This diagram is showing the contractile properties of tibialis anterior muscle in the hindlimb unloading plus electrical stimulation (ES) and hindlimb unloading plus the combination of electrical stimulation and resistance isometric contraction (ES+IC) groups. In the ES group (A), tibialis anterior muscle was stimulated without ankle joint fixation, an isotonic contraction was occurred. In the ES+IC group (B), ankle joint was fixed at 90° by a removable plaster cast during the electrical stimulation, forceful isometric contraction was occurred.
Histological analysis
Twelve hours after the last stimulation, all animals were deeply anesthetized by an intraperitoneal injection of sodium pentobarbital of 83 mg/kg body weight, and then the tibialis anterior muscle was removed and weighted. Thereafter, the animals were sacrificed by overdose of sodium pentobarbital. The muscles were immediately frozen in acetone-cooled dry ice and stored at -70°C until analysis. Transverse sections of 15 μm thick were cut on a cryostat (CM-1510S, Leica Microsystems, Mannheim, Germany) from the middle part of the muscle belly in the tibialis anterior muscles at -5°C, and mounted on glass slides. For myofibrillar adenosine triphosphatase (ATPase) histochemistry, the sections were pretreated at pH 7.6 to categorize the muscle fiber as type I, IIA, or IIB, and then used to determine the composition of muscle fiber types and to measure cross-sectional areas of each muscle fiber type. A measuring field was set over the entire muscle cross-section for the muscle fiber type composition. At least 100 randomly selected cross-sectional areas of each muscle fiber type were investigated. A measurement of the sections was calculated by using the ImageJ software program (NIH, Maryland, USA).

Western blot protocol
Approximately 50 mg of each tibialis anterior muscles was homogenized in buffer (10 mM NaCl and 50 mM Tris-HCl, pH 7.8), containing a protease inhibitor cocktail (1:200, Sigma, MO, USA). Total protein concentration was measured using a protein assay kit (BioRad, CA, USA). Twenty-five micrograms of protein was loaded on a 12.5% SDS-polyacrylamide gel, and after 90 min of electrophoresis at 120 V, the proteins were transferred to a PVDF membrane for 90 min at 200 mA. Following over night blocking step in 5% skimmed milk in PBST, the membrane was incubated in anti-HSP72 (1:10000 in PBST, SPA-810, Stressgen, Victoria, Canada) overnight at 4°C, and after incubation in goat anti-rabbit secondary antibody (1:10000) for 1 h, the proteins were detected using a chemiluminescent (ECL, Amersham, NJ, USA). Images were analysed with an image reader (LAS-1000, Fujifilm, Tokyo, Japan), and then the digitized signals were quantified using SCIENCE LAB IMAGE SYSTEM software (version 4.22).

Statistical analysis
The data are expressed as mean ± SEM. Significant differences between the four experimental groups were analyzed by using a one-way analysis of variance followed by Scheffe’s post hoc test. The statistically significant level was set at p < 0.01.

RESULTS

Muscle wet weight
The mean values of the wet weight of the tibialis anterior muscle were 451 ± 5 mg in the Cont group, 353 ± 8 mg in the HU group, 405 ± 5 mg in the ES group, and 434 ± 3 mg in the ES+IC group (Figure 2). The values in the HU and ES groups were significantly less than that in the Cont group (p < 0.01), whereas there were no significant differences between the Cont and ES+IC groups. The values in the ES and ES+IC groups were significantly larger than that in the HU group (p < 0.01). The value in the ES+IC group was appeared to be larger than that in the ES group and attenuated toward that in the Cont group.

Distribution of muscle fiber types and cross-sectional area
The ATPase histochemistry revealed that the tibialis anterior muscles were composed of type I (4.7%), type IIA (11-17%), and type IIB (76-83%) fibers (Figure 3), and there were no significant difference between all groups. The mean values of the cross-sectional area of the type I fibers in the Cont, HU, ES, and ES+IC groups were 1067 ± 11, 822 ± 11, 1014 ± 12, and 1091 ± 11 μm², respectively (Figure 4A). The values in the HU and ES groups were significantly less than that in the Cont group (p < 0.01), whereas there were no significant differences between the Cont and ES+IC groups. The values in the ES and ES+IC groups were significantly larger than that in the HU group (p < 0.01). The value in the ES+IC group was significantly larger than that in the ES group (p < 0.01). These relations among four groups were
also confirmed in the type IIA (Figure 4B) and IIB (Figure 4C) fibers. The values in the HU and ES groups were significantly less than those in the Cont group \( (p < 0.01) \), whereas the value in the ES+IC group was maintained at Cont level. The values in the ES and ES+IC groups were significantly larger than that in the HU group \( (p < 0.01) \), and the ES+IC group was significantly larger than the ES group \( (p < 0.01) \).

**HSP72 protein level**

There was no obvious change in the HU group compared to the Cont group. On the other hand, the HSP72 expression in the ES and ES+IC groups increased by 489% and 474% over the Cont group, respectively \( (p < 0.01; \text{Figure 5A}) \). There were no significant differences between the ES and ES+IC groups. A representative western blot for HSP72 is shown in Figure 5B.

**DISCUSSION**

The novel findings of the present study include the combination of electrical stimulation and forceful isometric contraction sufficiently prevented a decrease of the muscle mass in...
unloading muscle, and the cross-sectional areas in all fiber types were maintained at Cont levels in the electrical stimulation plus forceful isometric contraction group. Although some studies have reported that electrical stimulation without resistance isometric contraction was effective on muscle atrophy, it was not enough to maintain at control level [8, 10, 11, 14]. Therefore the results in the present study suggest that the combination of electrical stimulation and resistance isometric contraction is more effective than usual electrical stimulation to prevent muscle atrophy. It was considered that the difference of muscle contractile force between with and without joint fixation during electrical stimulation caused the difference of the preventive effect on muscle atrophy. In the present study, the muscle contractile force evoked by the electrical stimulation with resistance isometric contraction (ES+IC) group was approximately three times larger than that in the electrical stimulation without resistance isometric contraction (ES) group. Generally, resistance exercise needing much muscle contractile force is acknowledged to be effective increasing muscle strength [31], and causes hypertrophy in all muscle fiber types [32]. An isotonic contraction was occurred in the tibialis anterior muscles by electrical stimulation in the ES group, whereas resistance isometric contraction was occurred by joint fixation in the ES+IC group. It is said that peak force by isometric contraction is larger than that by isotonic contraction [33], and isometric contraction exercise results in greater protection from muscle atrophy [34, 35]. Therefore, in the present study suggested that the preventive effect of electrical stimulation on muscle atrophy depends on the amount of load to the muscle due to with and without resistance isometric contraction produced by joint fixation. The level of HSP72 expression was enhanced by electrical stimulation, but there was no significant difference between the ES and ES+IC groups. For the HSP72 response to exercise, Liu et al. [28] reported that it was induced by resistance exercise, but not by endurance exercise with low load. It has been also reported that resistance exercise-induced HSP72 depend on the exercise intensity [28]. Because muscle contractile force evoked by electrical stimulation in the ES+IC group was larger than that in the ES group, it was expected that the ES+IC group might have greater increased expression of HSP72 than the ES group, but it was not in fact. There is a possibility that equally increase of HSP72 between two electrical stimulation groups was associated with intensity-related threshold. Milne et al. [36] investigated alteration of HSP72 expression at several exercises, demonstrated that the expression of HSP72 did not exhibit intensity-dependent linear response and attained upper limitation after threshold. Because the intensities of electrical stimulation in both the ES and ES+IC groups were over the threshold, there might have been no difference of the HSP72 expression between the ES and ES+IC groups. Skeletal muscle atrophy induced by unloading is associated with decreased protein synthesis [37] and increased protein degradation [38, 39]. It has been reported that HSP72, molecular chaperone facilitate folding of nascent polypeptide chain, is involved in protein synthesis in cells [40]. Although muscle atrophy was prevented effectively with the combination of electrical stimulation and forceful resistance contraction, there was no significant difference in HSP72 expression between electrical stimulation with and without resistance isometric contraction. Therefore, it was speculated that protein degradation system was related rather than protein synthesis system including HSP72.

CONCLUSION

In the present study, hindlimb unloading for seven days resulted in the tibialis anterior muscle atrophy. Electrical stimulation could prevent it atrophy, and the combination of electrical stimulation and resistance isometric contraction was more effective than electrical stimulation without resistance. It is still unclear what mechanisms did enhance the preventive effect of electrical stimulation on muscle atrophy and needs further study, but the combination of electrical stimulation and resistance isometric contraction is available for effective therapeutic intervention.

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DECLARATION OF INTEREST

There is no conflict of interest.

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