

Original Article

Influence of exercise intensity on atrophied quadriceps muscle in the rat

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Abstract [Purpose] The aim of this study was to determine the effect of resistance training on atrophied skeletal muscle in rats based on evidence derived from physical therapy. [Subjects and Methods] Rats were forced to undergo squats as resistance training for 3 weeks after atrophying the rectus femoris muscle by hindlimb suspension for 2 weeks. The intensity of resistance training was adjusted to 50% and 70% of the maximum lifted weight, i.e., 50% of the one-repetition maximum and 70% of the one-repetition maximum, respectively. [Results] Three weeks of training did not alter the one-repetition maximum, and muscle fibers were injured while measuring the one-repetition maximum and reloading. The decrease in cross-sectional area in the rectus femoris muscle induced by unloading for 2 weeks was significantly recovered after training at 70% of the one-repetition maximum. The levels of muscle RING-finger protein-1 mRNA expression were significantly lower in muscles trained at 70% of the one-repetition maximum than in untrained muscles. [Conclusion] These results suggest that high-intensity resistance training can promote atrophic muscle recovery, which provides a scientific basis for therapeutic exercise methods for treatment of atrophic muscle in physical therapy.

Key words: Muscle atrophy, Quadriceps muscle, Resistance training

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INTRODUCTION

Skeletal muscle atrophy caused by disuse, unloading, weightlessness, poor nutrition and disease leads to decreased muscle fiber area and muscle strength. The preferred treatment for skeletal muscle atrophy in the clinic involves physical therapy. By contrast, skeletal muscle hypertrophy caused by mechanical stimulation such as resistance training and loading with terrestrial gravitation results in an increase in muscle fiber area and muscle strength. This enlargement in the morphological size of the muscle is mainly due to an increase in the size of muscle fibers^{1, 2)}. Thus, skeletal muscle is capable of adapting to the environment. Consequently, when skeletal muscle atrophies due to disuse caused by local immobilization or unloading during treatment after an external injury, physical therapy is applied to encourage early recovery.

Resistance training is the standard method of inducing

muscle hypertrophy in humans and other animals³⁻⁵⁾, and it is important for physical therapy to generate muscle hypertrophy and to intensify muscle power. Exercise with relatively high external resistance equivalent to 70–85% of the one-repetition maximum (1RM) is generally recommended for increasing strength via resistance training. Dons et al.⁶⁾ reported that dynamic muscle strength increases in response to lifting weight at 80% 1RM. High-intensity resistance training is effective in humans from the viewpoint of the rate of muscle protein synthesis^{7, 8)}. Tamaki et al.⁹⁾ showed that resistance training with 65–75% 1RM for 12 weeks induced muscle hypertrophy in rats. Suetta et al.¹⁰⁾ also reported that retraining with a 65–70% 1RM load for 4 weeks recovered the strength of the human quadriceps muscle with disuse atrophy induced by immobilization for two weeks. Further, Campbell et al.¹¹⁾ indicated that 3 weeks of resistance training at 80% 1RM after unilateral lower limb suspension for 3 weeks can temporarily recover a decrease in isometric torque, muscle volume, fascicle length, and physiological cross-sectional area in the quadriceps muscle of young men. However, the effect of resistance training has not been investigated with respect to the repetition maximum concept when targeting an atrophic muscle in an animal model of muscle atrophy caused by disuse.

The ubiquitin-proteasome system contributes to a specific proteolysis mechanism and is involved in muscle atrophy,

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as is the autophagy-phagosome system. In this system, myostatin (involved in the control of muscle size), muscle atrophy F-box protein (atrogin-1; plays a role in the proteolysis of myogenic differentiation 1 (MyoD)), eukaryotic translation inhibition factor 3 subunit f (eIF3f), desmin and vimentin, and muscle RING-finger protein-1 (MuRF1; involved in the ubiquitination of myosin heavy chain, myosin light chain, actin, troponin I, and myosin binding protein C) are all expressed during muscle atrophy via the ubiquitin-proteasome system^{12–19}). Resistance training decreases the mRNA expression of myostatin, atrogin-1, and MuRF1^{20,21}).

Expression of insulin-like growth factor-1 (IGF-1) mRNA activates the Akt/mammalian target of rapamycin (mTOR)/p70S6 kinase (p70S6 K) pathway via eccentric exercise and increases the size of the rat gastrocnemius muscle²²). Resistance training during the initial stages of unloading-induced muscle atrophy can suppress this decrease in muscle size by increasing levels of IGF-1 mRNA and the activity of anabolic signaling intermediates such as p70S6 K in rats²³). However, chronic resistance training increases IGF-1 mRNA expression and suppresses the decrease in muscle size, although such training suppresses Akt/mTOR phosphorylation in aged rats⁴). Thus, muscle atrophy and hypertrophy are controlled by highly specific and complex molecular mechanisms.

Consequently, disuse atrophy is treated by resistance training and/or physical stimulation during physical therapy. However, the one-repetition maximum is an important basis of training intensity applied for resistance training in physical therapy, although many contrasting studies suggest that it is not the optimal value for recovering the strength of atrophic muscle. Therefore, we examined the effect of resistance training using the repetition maximum concept on recovery of disused atrophic muscle in physical therapy.

SUBJECTS AND METHODS

Animal care and handling proceeded according to the Guidelines for the Care and Use of Laboratory Animals at Kanazawa University, and the Committee on Animal Experimentation at Kanazawa University approved all protocols. Thirty-six male Wistar rats aged 9 weeks (Kiwa Laboratory Animals Co., Ltd., Misato-cho, Wakayama, Japan) were housed in a temperature-controlled room (20–24 °C) with a 12-h light/12-h dark cycle and access to laboratory chow and water ad libitum. We determined the histological effect of one regime of squat exercise at 1RM in six rats after 5 weeks. Thirty rats were then randomly allocated into the following groups (n = 6 per group): the control, unload (hindlimb unloaded as described by Morey-Holton et al.²⁴) for 2 weeks), reload (unloading for 2 weeks followed by reloading for 3 weeks without training), 50% 1RM (unloading for 2 weeks followed by reloading for 3 weeks with training at 50% 1RM) and 70% 1RM (unloading for 2 weeks followed by reloading for 3 weeks with training at 70% 1RM groups).

Both resistance training and 1RM measurements were achieved using the modified animal squat training device developed by Krisan et al.²⁵) We used an electric stimulator (Nihon Kohden, Tokyo, Japan) to stimulate the soles of both feet with 5 V of electricity, which caused the rats to react in a manner resembling plyometric squats, thus generat-

ing substantial concentric and eccentric contraction of the quadriceps muscle. The 1RM in the reload, 50% 1RM and 70% 1RM groups was measured after 1 day of reloading, and training at the 50% 1RM and 70% 1RM intensities was started as follows: 14 repetitions/set, three sets/day, three times/week at 50% 1RM and 10 repetitions/set at 70% 1RM, with 1-minute intervals between sets. The resistance weight comprised 10 g or 25 g stainless steel plumbs.

The 1RMs of the reload, 50% 1RM, and 70% 1RM groups were measured after reloading for 3 weeks and were also measured at 2 days before tissue extraction. After euthanasia, the rectus femoris muscle was extracted from the right hindlimbs of all rats, placed in Tissue-Tek O.C.T. compound (Sakura Finetek Japan, Tokyo, Japan) and frozen in isopentane cooled in liquid nitrogen. The frozen tissues were cut using a CM-41 cryostat (Sakura Finetek) into 10- μ m-thick transverse sections for myosin ATPase and hematoxylin and eosin (HE) staining and then dried for 2 hours at room temperature. Type I and II fibers in transverse sections were stained with myosin ATPase at pH 10.4. Sections were visualized using a Biozero BZ-8000 microscope (Keyence, Osaka, Japan). Faint and intensely stained muscle fibers were classified as type I and II fibers in five regions per section, and the ratios (%) of type II fibers among the totals were calculated in the control group. Cross-sectional areas of 200 unclassified myofibers per muscle were digitized and assessed using the ImageJ image analysis software.

Samples of muscle tissues from the reload, 50% 1RM, and 70% 1RM groups were soaked in RNeasy RNA Stabilization Reagent (Qiagen, Tokyo, Japan) overnight at 4 °C and stored at –80 °C. Total RNA was then isolated from the stored muscle samples using an RNeasy® Fibrous Tissue Mini Kit (Qiagen) according to the manufacturer's protocol. The RNAs were reverse transcribed using PrimeScript™ 1st strand cDNA Synthesis Kits (Takara Bio Inc., Otsu, Shiga, Japan). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using a LightCycler® ST300 (Roche Diagnostics, Tokyo, Japan) and SYBR Premix Ex Taq™ II (Takara Bio Inc.) with the following primer sets (Nihon Gene Research Laboratories Inc., Sendai, Japan): 5'-CCAAGGGGCTTTACTTCAAC-3' and 5'-TGGAACGAGCTGACTTTGTA-3' for IGF-1, 5'-CATTACCATGCCTACCGAGTC-3' and 5'-GGCCTTACCCTTTGTTGTACTG-3' for myostatin, 5'-ATGAGAAAAGCGGCACCT-3' and 5'-TGGTTTTGCTGTTCTGTATGTC-3' for atrogin-1, 5'-GGAGGAGGAGGATCAAGA-3' and 5'-CTGAGCAGAGAGAAGACACAC-3' for MuRF1, 5'-AACGGGAAACCCATCACCA-3' and 5'-CGGAGATGATGACCCCTTTG-3' for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The qRT-PCR conditions were as follows: 10 s at 95 °C and then 40 cycles of 5 s at 95 °C and 20 s at 60 °C, and this was followed by melting curve analysis. The reaction conditions were optimized for efficient standard curve analysis. The specificity of each primer set was judged based on the amplification of a single product determined as a single peak at the appropriate melting temperature. Expression levels of IGF-1, myostatin, atrogin-1 and MuRF1 mRNA were normalized by subtracting the corresponding GAPDH mRNA. The relative contents of the

respective mRNAs in trained muscles were compared with reloaded muscle.

Data are presented as means \pm standard deviation (SD). Differences in body weight and cross-sectional areas between groups were assessed using a one-way analysis of variance followed by Tukey's post hoc test. Differences between trained groups and the reload group were detected by Dunnett's or Steel's multiple comparison tests. Values of $p < 0.05$ were considered to indicate statistical significance in all tests.

RESULTS

The 1RM in normal rats was $1,842.7 \pm 144.1$ g, and the ratio of the 1RM to body weight was 4.3 ± 0.2 . Muscle fiber injury in the rectus femoris muscle induced by measuring the 1RM was sparse (Fig. 1). The body weights of the rats in the control, unload, reload, 50% 1RM and 70% 1RM groups were 332.0 ± 6.6 g, 285.5 ± 12.6 g, 380.5 ± 25.8 g, 362.0 ± 26.2 g and 395.0 ± 17.9 g, respectively. The control group weighed significantly less than the reload, 50% 1RM and 70% 1RM groups, and the unload group weighed significantly less than all other groups (Table 1). The cross-sectional areas of the rectus femoris muscle, which were initially decreased by hindlimb unloading for two weeks, showed significant recovery in the 70% 1RM training group (Table 1).

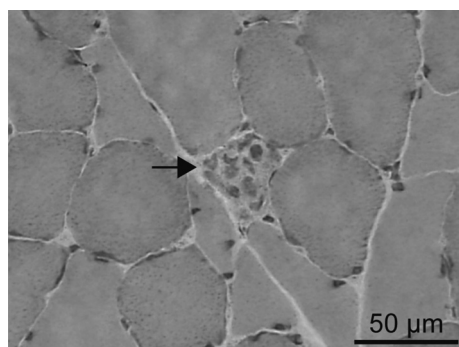


Fig. 1. Hematoxylin and eosin staining of rectus femoral muscles in 14-week-old rats after measuring the 1RM

Arrowhead: sparse inflammatory cell infiltration

The 1RM of rats at the end of 3 weeks of training after reloading was significantly lower than that of the reload group training at 50% 1RM (Table 2). However, the difference in the ratio of the 1RM to body weight did not significantly differ among the three groups (Table 2). The rectus femoris muscles in the control group consisted almost entirely ($97.0 \pm 1.0\%$) of type II fibers with high levels of ATPase activity, and type I fibers were mostly located in the periphery of the muscle (Fig. 2A1–3). The frequency distribution in cross-sectional areas showed global muscle fiber atrophy in unloaded muscles, increased numbers of middle dimension muscle fibers in reloaded muscles, and 50%1RM trained muscles and curves with double peaks in 70% 1RM trained muscles (Fig. 2B).

Next, we assessed the effects of unloading, reloading, and training on the rectus femoris muscle using HE staining (Fig. 3). Though muscle fiber dimension decreased temporarily by unloading, it was recovered by hindlimb unloading for 2 weeks (Fig. 3; unload). Fibers with central nuclei were generated by reloading (Fig. 3; reload and 50% 1RM). Some small fibers in the 70% 1RM trained muscles split and swelled (Fig. 3; 70% 1RM). The expression of IGF-1, myostatin and atrogin-1 mRNA was essentially undetectable in untrained and 50% 1RM and 70% 1RM trained muscles, whereas MuRF1 mRNA expression was significantly downregulated in 70% 1RM trained muscles to the level of untrained muscles (Table 3).

DISCUSSION

In the present study, measurement of the 1RM resulted in injury to muscle fibers in the rat rectus femoris muscle. A previous study reported similar findings, that is, that squat training leads to the appearance of fibers with central nuclei, split and new small-diameter fibers in the rat plantaris muscle, and increased blood levels of creatine kinase activity⁹. Despite using different target muscles, these data suggest that brief high intensity exercise such as those performed at the 1RM cause muscle fiber injury in rats.

To evaluate the effect of resistance training during reloading for 3 weeks on atrophied rectus femoris muscle that had atrophied as a result of unloading, we measured the 1RMs of the reload, 50% 1RM and 70% 1RM groups. Previous studies reported that squat training for 12 weeks increases

Table 1. Body weight of rats and cross-sectional areas of the rat rectus femoris muscle

	Control	Unload	Reload	50%1RM	70%1RM
Body weight (g)	$332.0 \pm 6.6^\dagger$	$285.5 \pm 12.6^*$	$380.5 \pm 25.8^{***}$	$362.0 \pm 26.2^{**}$	$395.0 \pm 17.9^{***\dagger}$
Cross-sectional areas (μm^2)	$3,326.3 \pm 431.0^{**}$	$2,083.6 \pm 375.5$	$2,665.5 \pm 333.3$	$2,816.3 \pm 327.7$	$2,916.3 \pm 549.4^*$

* $p < 0.05$ and ** $p < 0.01$ vs. Unload; $^\dagger p < 0.01$ vs. Control

Table 2. One-repetition maximum of rats followed by reloading and training for 3 weeks

	Reload	50%1RM	70%1RM
One-repetition maximum (g)	$1,876 \pm 150$	$1,628 \pm 169^*$	$1,756 \pm 136$
One-repetition maximum/body weight (g/g)	5.0 ± 0.6	4.5 ± 0.7	4.5 ± 0.5

* $p < 0.05$ vs. Reload

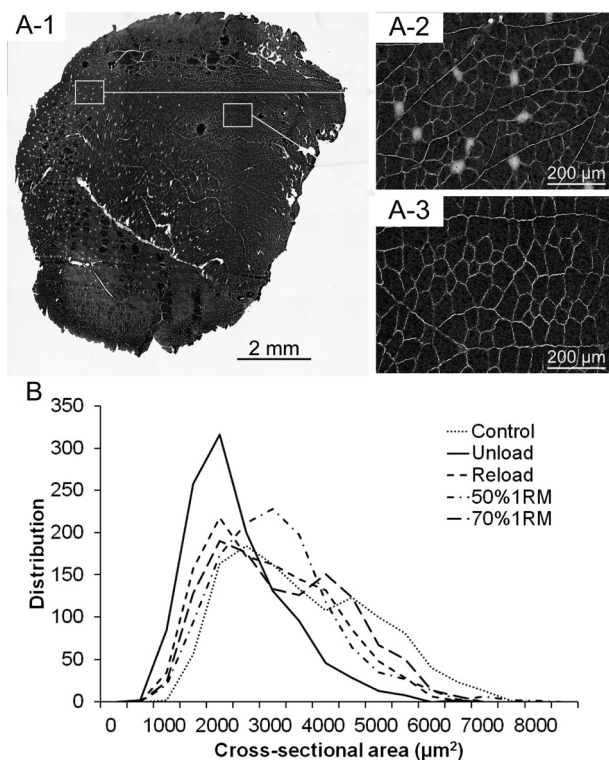


Fig. 2. Histological analysis of rat rectus femoris muscle Type I and II fibers are intensely and faintly stained, respectively, with myosin ATPase in the control group (A). The rectus femoris muscle is largely comprised of type II fibers in the control group (A-1); type I fibers are mostly located in periphery of the rectus femoris muscle (A-2), and regions without type I fibers are evident (A-3). Histogram showing the distribution of cross-sectional area in the control, unload, reload, 50% 1RM, and 70% 1RM groups (B)

Table 3. Real-time PCR analysis of IGF-1, myostatin, atrogen-1 and MuRF1 mRNA expression in rat rectus femoris muscles 3 weeks after reloading

	Reload	50%1RM	70%1RM
IGF-1	1.0 ± 0.3	1.2 ± 0.4	1.3 ± 0.3
Myostatin	1.0 ± 0.4	1.0 ± 0.3	1.0 ± 0.3
Atrogen-1	1.0 ± 0.5	0.9 ± 0.2	0.9 ± 0.3
MuRF1	1.0 ± 0.2	1.3 ± 0.7	0.6 ± 0.2*

*p < 0.05 vs. Reload

the 1RM^{9, 25}). However, the effect of short-term training for 3 weeks was not investigated. There was no change in the 1RM in the present study, although measurement of the 1RM, which was performed once, and reloading caused damage to the rectus femoris muscle (Figs. 1 and 3). Reloading followed by unloading reportedly caused muscle fiber injury in the rodent soleus and plantaris muscles^{26, 27}, and Flann et al.²⁸ reported that acute training induces pain in the human quadriceps muscle. Consequently, improving muscle power over the short term seems difficult, particularly in

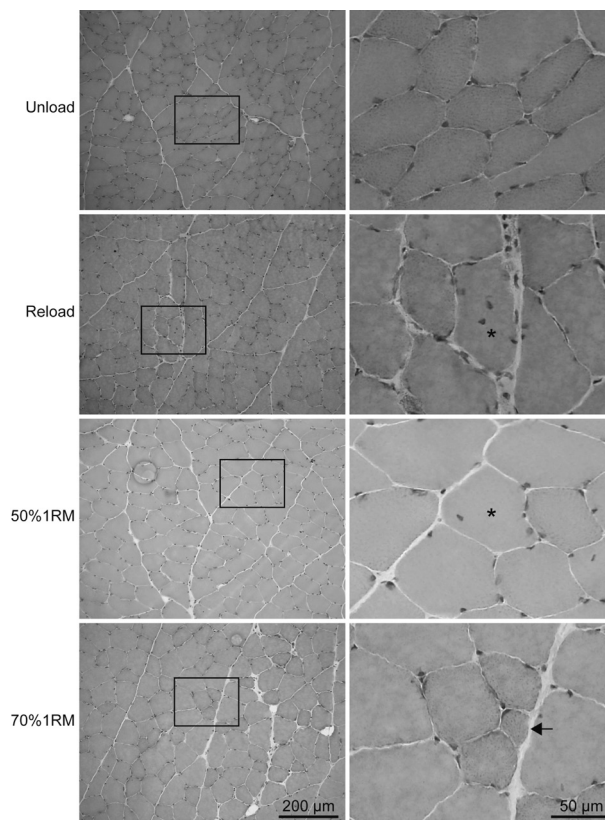


Fig. 3. Hematoxylin and eosin staining of cross sections of the rectus femoris muscles

The left and right columns show overview and pathognomonic findings in unload, reload, 50% 1RM, and 70% 1RM groups, respectively. The right line expanded a square of the left line, respectively. *Central nuclei fibers in the reload and 50% 1RM groups. Arrowhead: small muscle fibers in the 70% 1RM group

atrophic muscles.

The density of oxidative type I fibers is high in the soleus muscle but only accounts for approximately 7% of the rectus femoris muscle²⁹). In the present study, this ratio was $3.0 \pm 1.0\%$. However, the rats were 16–18 weeks old in the study by Diaz-Herrera²⁹), whereas those in the present study were 11 weeks old. The ratio of type I fibers generally increases with age, which may explain these discrepant findings.

Unloading for 2 weeks causes atrophy in most rat hindlimb muscles, including the quadriceps³⁰). Conversely, resistance training in non-artificially manipulated rats using ladder and jumping into water for 8 and 12 weeks increased fiber cross-sectional areas in the flexor digitorum longus and plantaris muscles^{31, 32}). Many previous studies have reported that recovery of a rat soleus muscle with atrophy induced by hindlimb suspension is accelerated by reloading^{27, 33–36}) and running^{37, 38}). In the present study, the decreases in body weight and muscle fiber cross-sectional areas induced by unloading for 2 weeks were not recovered by reloading for 3 weeks. However, the cross-sectional area was significantly increased in the 70% 1RM group compared with the unload group. Further, the distribution of cross-sectional areas showed an increased number of small fibers in the unload

group and an increased number of bimodal large fibers in the 70% 1RM group. These findings indicate that 70% 1RM training increased the numbers of muscle fibers that had previously been atrophic. We measured the total cross-sectional area without discriminating between type I and II fibers due to deflection of the position of type I fibers, although type I and II fibers were separated by ATPase staining. Consequently, the relationship between training intensity and muscle fiber types is unclear.

Previous studies have found that resistance training can injure muscle fibers and lead to elevated levels of regenerative myogenic regulatory factor in skeletal muscle^{9, 39}). In the present study, pathological alterations such as central nuclei and necrosis fibers were evident after measuring the 1RM and resistance training for 3 weeks. However, secondary muscle damage after exercise that is required for recovery is accelerated^{40–42}). Muscle cell apoptosis and satellite cell activation occur simultaneously during muscle unloading and reloading; thus, an alternative process involves complete fiber degradation and cell death, followed by *de novo* synthesis of new fibers via satellite cell activation^{43, 44}). Small split myofibers that appear after high-intensity exercise arise through pinching off a small segment of parent muscle fiber or invagination of the sarcolemma deep in muscle fibers in a plane parallel to sarcomeres⁴⁵). We also found evidence of split fibers, indicating that 70% 1RM training provided sufficient high-intensity stimulation to recover the atrophic rat rectus femoris muscle.

For evaluating the effect of resistance training during reloading for three weeks on rectus femoris muscle that had atrophied as a result of unloading, we assessed mRNA levels of IGF-1, myostatin, atrogen-1, and MuRF1 in the reload, 50% 1RM and 70% 1RM groups. Resistance training did not alter the expression of IGF-1, myostatin, or atrogen-1 mRNA, whereas MuRF1 mRNA expression was decreased by 70% 1RM training compared with no training. The muscle-specific ubiquitin E3-ligase MuRF1, which is part of the ubiquitin proteasome pathway used for protein degradation during muscle atrophy⁴⁶), is upregulated during muscle atrophy⁴⁷). Kobayashi et al.⁴⁸) reported that chronic isometric training decreases MuRF1 and atrogen-1 protein levels in the gastrocnemius muscles of Sprague-Dawley but not Wistar rats, indicating that Wistar rats are less sensitive to resistance training. Louis et al.⁴⁹) demonstrated an acute increase in MuRF1 mRNA expression after resistance training in humans. By contrast, Zanchi et al.²¹) reported that chronic resistance training for 12 weeks decreased MuRF1 and atrogen-1 levels in the plantaris muscles of Wistar rats. These findings suggest that MuRF1 is upregulated by muscle atrophy and acute resistance training and downregulated by chronic resistance training. Thus, MuRF1 is apparently downregulated when protein breakdown decreases. Similarly, MuRF1 upregulation may participate in removing useless tissue during muscle atrophy and in recycling in conjunction with tissue reconstruction during acute resistance training. In the present study, the decrease in MuRF1 expression induced by 70% 1RM training inhibited protein breakdown after muscle disuse.

In summary, our data suggest that high-intensity resistance training is an effective physical therapy method for

treatment of atrophic muscles. However, reloading and/or overloading of atrophic muscle induced by muscle fiber injury may also cause inflammation and pain. Additional multiphasic studies are required to determine the appropriate level and duration of high-intensity training for physical therapy of atrophic muscle in humans.

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