Effect of Ultrashort Wave on Joint Dysfunction and Muscle Atrophy in a Rabbit Model of Extending Knee Joint Contracture: Enhanced Expression of MyoD

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Abstract

Background As a common clinical disease, the incidence of joint contracture which is characterized by the reduction of range of motion (ROM) in the active or passive state of the joint has increased in recent years. This study was to investigate the effects of ultrashort wave on joint dysfunction and muscle atrophy in a rabbit model of extending knee joint contracture and its mechanism. Methods 35 rabbits underwent unilateral immobilization of a knee joint at full extension to cause joint contracture, and 5 rabbits were used for the control group. After 8 weeks immobilization, 35 rabbits were randomly divided into the following seven groups: I-8, R-1, R-2, R-4, T-1, T-2, and T-4. In the Group R-1, R-2 and R-4, the rabbits were experienced one, two, and four weeks self-recovery. In the Group T-1, T-2, and T-4, the rabbits were experienced one, two, and four weeks ultrashort wave treatment. The effect of self-recovery and ultrashort wave treatment on joint dysfunction and muscle atrophy was assessed by measuring the degree of total and myogenic contracture, evaluating the cross-sectional area (CSA) of rectus femoris and assessing the protein levels for MyoD. Results A tendency toward reduced the degree of total and myogenic contracture was observed after self-recovery and ultrashort wave treatment. A tendency toward increased the CSA of rectus femoris and the protein levels for MyoD was observed after self-recovery and ultrashort wave treatment. The ultrashort wave treatment led a better efficacy than self-recovery against the total and the myogenic contracture, the CSA and the protein levels for MyoD of rectus femoris. Conclusions Ultrashort wave ameliorates joint dysfunction and muscle atrophy via upregulating the expression of MyoD protein in a rabbit model of extending knee joint contracture.

Background

The range of motion (ROM) of knee joint is maintained by repeated daily movements.[1] The normal ROM is difficult to restore once lost even through surgical treatment, and immobilization is a major cause of knee joint contracture.[2] Two different structural components which contained myogenic contracture and arthrogenic contracture make contribution to joint contracture formation.[3] Studies on animal muscles have shown that passive extensibility depends on the size and length of the muscle fibers and the amount and arrangement of connective tissue in the muscle belly.[4] According to this, extending knee joint contracture can be treated by quadriceps stretching and joint mobilization at the department of rehabilitation medicine. In recent years, most studies are dedicated to improving arthrogenic contracture, but few researches are focused on improving myogenic contracture.

Muscle atrophy is an important part of myogenic contracture.[2] Sustained muscle atrophy which caused by loss of tissue protein because of decreased synthesis and increased degradation, an increase in the amount of intramuscular connective tissue, and the arrangement of collagen fibrils in the endomysium severely affects the function of knee joint.[5] During the process of muscle atrophy from immobilization in shortened position, the mass of muscle was reduced.[6] Meanwhile, the length and cross-sectional area (CSA) of myofibers are contractible compared with normal.[2] MyoD is one of the earliest markers of myogenesis.[7] Recent evidence demonstrate that increasing ubiquitin-proteasome-dependent proteolysis

is closely related to muscle atrophy.[8,9] As a key point of this proteolytic pathway, myogenic differentiation (MyoD) which is one of myogenic regulatory factors (MRFs) plays a crucial role in inhibiting muscle proteolysis.[10]

The ultrashort wave which usually used to treat for soft tissue inflammation has a frequency of 30-300 MHz and a wavelength of 10-1 m. In our previous study of effect of stretching combined with ultrashort wave on joint function, we found that the ultrashort wave therapy can reduce joint capsule fibrosis.[11] Interestingly, we also found that the ultrashort wave therapy can reduce muscle atrophy effectively in our subsequent muscle tissue analysis, so we completed the following study. Our objective in this study was to investigate the effects of ultrashort wave on joint dysfunction and muscle atrophy in a rabbit model of extending knee joint contracture and its mechanism.

Methods

Animals

This experimental protocol was approved by the Institutional Animal Care and Use Committee of Anhui Medical University. A total of 40 male skeleton mature New Zealand white rabbits were used in this study (Anhui Medical University Experimental Animal Center, Hefei, China, age 3-4 months, weight 2-2.5 kg). The rabbit which were maintained in 60 cm × 50 cm × 40 cm cages, were exposed to a 12-h light–dark cycle at an ambient temperature of 24 °C, then allowed free activities in cages and free access to water and food. All of them were anesthetized by ear intravenous administration of 30mg/kg sodium pentobarbital. Then 35 rabbits underwent unilateral immobilization of a knee joint at full extension using a plaster cast from groin to proximal toes as the previous research (Figure 1A), [11] and 5 rabbits were used for control group corresponding to C.

After 8 weeks immobilization, the plaster cast of the left knee joint was removed. Then 35 rabbits were randomly divided by random number table method into 7 groups, 5 rabbits in each group, corresponding to I-8, R-1, R-2, R-4, T-1, T-2, and T-4.

Intervention methods

In Group C, the rabbits did not undergo immobilization, and the rabbits only underwent 8 weeks immobilization in Group I-8. In addition, there were two intervention methods about self-recovery and ultrashort wave treatment. Specifically, Group R-1, R-2 and R-4 only underwent self-recovery for 1, 2, 4 weeks respectively. Relatively, Group T-1, T-2 and T-4 not only experienced self-recovery, but also experienced ultrashort wave treatment. For instance, each animal that needed to receive ultrashort wave treatment was placed on the floor and the experimental knee joint was exposed to the electrodes of ultrashort wave treatment equipment. After the equipment was preheated, the mode of the ultrashort wave treatment equipment was transferred to micro heat and the time of treatment was set to 15 mins, once a day (Figure 1B).

Tissue sampling and H&E

At the end of each time point, the rabbits were euthanized with an excess of sodium pentobarbital. The left hind limb was dislocated at the left hip joint and completely removed. Then ROM of the left knee joint was measured by the joint motion measuring instrument as the previous experiment. Later, two muscle tissues of 1 cm × 1 cm × 0.5 cm in size were cut in the middle of the rectus femoris. One of them was used for H&E staining, and the other was frozen in liquid nitrogen at -80 °C until histological analysis.

Rectus femoris were stained with Hematoxylin and Eosin (H&E), and the CSA of individual myofibers was photographed using Nikon TE2000-U inverted microscope (Nikon Corporation, Tokyo, Japan) and measured using Image-Pro Plus (IPP) 6.0 software (Media Cybernetics, Inc., Silver Spring, MD, USA). Six randomly selected fields of view were analyzed in each group.

Calculation of the total and the myogenic contracture

Like our previous study, ROM of the left knee joint was measured by the joint motion measuring instrument. [11,12] According to the method described by Trudel G,[13] we evaluated myogenic contracture caused by the muscular structures including their tendons and fascia, and arthrogenic contracture caused by the articular structures including bone, cartilage, synovium, capsule, and ligaments (Figure 2). The formulas used were as follows:

1. Decrement in ROM as a result of total contracture = ROM before myotomy (of the control knee) − ROM before myotomy (of the contractured knee).
2. Decrement in ROM as a result of arthrogenic contracture = ROM after myotomy (of the control knee) - ROM after myotomy (of the contractured knee).
3. Decrement in ROM as a result of myogenic contracture = Decrement in ROM as a result of total contracture − Decrement in ROM as a result of arthrogenic contracture.

Protein Extraction and Western Blot

The skeletal muscle samples were ground into powder with liquid nitrogen using a grinder and homogenized in RIPA buffer (Beyotime, China) containing protease inhibitors at 4 ℃. Homogenates were centrifuged at 12,000×g for 30 min three times at 4 ℃, and the resulting supernatants were collected. The protein concentrations were determined using the bicinchoninic acid method. Protein lysates were separated on a 10 % sodium dodecyl sulfate-poly-acrylamide electrophoresis gel and transferred onto polyvinylidene fluoride membranes (Millipore, USA). After being blocked with 5 % non-fat dry milk in Tris-buffered salineTween-20 at RT for 2 h, the membranes were incubated with mouse anti-MyoD mAb (dilution 1:600; BF0314, Affinity Biosciences, USA) at 4 ℃ overnight. On the second day, after being washed in TBST solution three times per 10 mins, membranes were incubated with peroxidase conjugated affinipure goat anti-mouse IgG-HRP (dilution 1:3000; S0002, Affinity Biosciences, USA) as the secondary antibody for 2 hours at room temperature. After being washed three times with TBST per 10 mins, the membranes were then detected with the enhanced chemiluminescence system according to the

manufacturer's instructions. The densities of bands were quantified using Image J software. The relative protein levels were calculated by comparison with the amount of beta-Tubulin (T0023, Affinity Biosciences, USA) as a loading control.

Statistical analysis

All data are expressed as the mean ± standard error of the mean (S.E.M.). The assumptions of normality of data and homogeneity of variances between the groups was analyzed by SPSS 21.0 (Chicago, IL, USA). Differences in the total contracture, the myogenic contracture, the CSA, and the average protein levels for MyoD between the Group R and Group T at each recovery time point were assessed using Student's t-test. The significant difference between the Group R and Group T at the same time point was measured at 95% CI not overlapping zero. One-way analysis of variance (ANOVA) and the Tukey-Kramer test were performed to examine differences in the total contracture, the myogenic contracture, the CSA, and the average protein levels for MyoD among the time points.

Results

All of the rabbits survived throughout the immobilization and recovery period. Neither prolonged edema nor acute inflammation was observed in any rabbit.

The total and the myogenic contracture

The total contracture and the myogenic contracture were significantly reduced in Group T as compared with that in Group R, at the same time point ($P < 0.05$, Table 1). One-way ANOVA and the Tukey-Kramer test applied to the resulting curves indicated that both the total contracture and the myogenic contracture of Group T and Group R were decreased with the recovery time extension ($P < 0.05$, Figure 3).

Morphological changes of the rectus femoris

The CSA were significantly reduced in Group T as compared with that in Group R, at the same time point ($P < 0.05$, Table 1). One-way ANOVA and the Tukey-Kramer test applied to the resulting curves indicated that the CSA of Group T and Group R were increased with the recovery time extension ($P < 0.05$, Figure 4).

Protein Extraction and Western Blot

The average protein levels for MyoD were significantly reduced in Group T as compared with that in Group R, at the same time point ($P < 0.05$, Figure 5). One-way ANOVA and the Tukey-Kramer test applied to the resulting curves indicated that the average protein levels for MyoD of Group R were increased with the recovery time extension, and the average protein levels for MyoD of Group T were increased with the recovery time extension except at the first week. ($P < 0.05$, Figure 5).

Discussion

In this study, the total contracture and the myogenic contracture in the immobilized knee have significantly increased after 8 weeks immobilization. This result suggested that there was serious knee joint dysfunction. In the period of recovery, the relative contributions of the total contracture and the myogenic contracture exhibit different tendencies between the Group T and Group R. In general, Group T showed a more obvious downward trend in the total contracture and the myogenic contracture by comparing Group R. In addition, Group T also showed a more obvious upward trend in the CSA by comparing Group R. In agreement with the decrease in the total contracture and the myogenic contracture, which was parallel to the increase of the CSA during remobilization in the rectus femoris. Muscle mass can influence the self-inertia of a joint.[14] Passive extensibility is influenced by the size and length of muscle fibers and the amount and arrangement of the connective tissues of the muscle belly.[15] This meant that the muscle length and CSA of the rectus femoris in Group T were lager than Group R, suggesting that ultrashort wave was effective in treating myogenic contracture and muscle atrophy by ameliorating the muscle extensibility. In the Figure 4A d-f, it was unexpected but reasonable that the connective tissue of the rectus femoris seemed to be decreased, because our previously study also proved that ultrashort wave therapy reduced joint capsule fibrosis by down-regulating the protein levels for TGF-β1.[11] Unfortunately, the extensibility of rectus femoris cannot obtain due to the restriction of equipment conditions.

MyoD plays a widely implicated role in the ubiquitin–proteasome system.[16] Previous research used nandrolone (ND) to prevent disuse muscle atrophy in mice and found that MyoD expression was increased in ND-treated mouse soleus muscle, which slowed down the speed of the reduction for soleus muscle mass and protein content.[10] In addition, previous research showed that hamstring flexibility can be greatly improved by shortwave combined with prolonged stretching.[17] It was meaningful for the treatment, however, the pathological changes in muscle wasn't observed in this study. Like these research, we also found that ultrashort wave treatment can result in the reduction of the protein levels for MyoD more effectively compared with self-recovery, which was consistent with the effects on the myogenic contracture and the CSA, indicating that ultrashort wave treatment may partly influence the expression of MyoD and then ameliorate joint dysfunction. Interestingly, the average protein levels for MyoD in Group R-1 and Group T-1 expressed separately declining and stable trend by comparing with Group I-8. This phenomenon was like the previous research. The worsening of tibialis anterior muscle atrophy during recovery was also occurred, and they considered that it correlated with enhanced connective tissue area, proteolysis, and apoptosis.[18]

It is common that immobilization causes significant joint contracture and muscle atrophy, however, there are few related treatments. Muscle atrophy is of major clinical importance and is a critical issue in terms of healthcare costs. Preventing activity of the ubiquitin–proteasome system is now available in animal models of muscle atrophy, but few drugs of proteasome inhibitors can be used in clinical since the proteasome inhibitors may also be of interest independently of the role of ubiquitin–proteasome system in myofibrillar proteolysis.[19,20] Ultrashort wave is a common therapy in the department of rehabilitation

medicine and the department of physiotherapy, so may be valuable as it prevents joint contracture and muscle atrophy.

The reason for setting the control group (Group C) was that it was necessary for calculate total contracture and myogenic contracture by the ROM of knee joint in Group C. In addition, during the process of muscle atrophy from immobilization in shortened position, the mass of muscle was reduced obviously, so we selected rectus femoris in this model of extending knee joint contracture.

There are several limitations in our study that warrant attention. Only the protein levels for MyoD was measured in this study, and further experiments are needed to evaluate the protein levels for other molecules and enzymes, to understand which other mechanisms may also be active in this period. In addition, we also need to obtain the extensibility of rectus femoris to identify the reduction of muscle fibrosis.

Conclusions

We have established a rabbit model of extending knee joint contracture that better recapitulates the joint dysfunction and muscle atrophy of clinical patients. Ultrashort wave ameliorates joint dysfunction and muscle atrophy via upregulating the expression of MyoD protein in a rabbit model of extending knee joint contracture. Despite clear differences between humans and animals, animal studies are necessary to gain further insight into the mechanisms of treatment underlying extending knee joint contracture. Ultrashort wave is a common therapy in the department of rehabilitation medicine and the department of physiotherapy, so it may be valuable as it prevents joint contracture and muscle atrophy.

Abbreviations

cross-sectional area (CSA), range of motion (ROM), myogenic differentiation (MyoD), myogenic regulatory factors (MRFs), Hematoxylin and Eosin (H&E), analysis of variance (ANOVA), nandrolone (ND).

Declarations

Ethics approval and consent to participate

All procedures performed in this study involving animals were approved by the Institutional Animal Care and Use Committee of Anhui Medical University (20180621006).

Consent for publication

Not applicable.

Availability of data and material

The datasets used and analysed during the current study are available from the corresponding author on reasonable request. All data generated or analysed during this study are included in this published article. The manuscript, including related data, figures and tables have not been previously published and are not under consideration elsewhere.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

We declare, that all authors have made substantial contributions to the conception and design of the study, acquisition of data, analysis and interpretation of data. All authors drafted the article critically for important intellectual content and made substantial contributions to final approval of the version to be submitted. FW, QBZ, YZ, JL were involved in plaster fixation of rabbit model. FW, QBZ, HZZ, AYL operated all animals and retrieved samples from all animals. H&E and western blot experiments were performed by FW with the help of YHX. Data analysis was supported by FW, YL and YZ. All authors were involved in reading and approving the final manuscript.

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Tables

Table 1. Mean (S.D.) raw values of the total contracture, the myogenic contracture and CSA for the Group R and Group T by time.

Group	total contracture (°)	myogenic contracture (°)	CSA(μm^2)
C	0 ± 0	0 ± 0	/
I-8	97.5 ± 3.4	40.5±2.7	4202.0 ± 56.3
R-1	92.8 ± 2.5	35.0±2.2	4368.0 ± 56.5
R-2	85.8±1.7	29.3±3.0	4545.3 ± 50.2
R-4	79.5±3.0	26.2±2.8	4663.0 ± 83.9
T-1	87.3±4.1 *	32.3±1.7 *	4425.5 ± 56.7 *
T-2	77.5±1.3 #	26.3±1.3 #	4729.5 ± 125.1 #
T-4	70.5±1.3 &	22.8±1.0 &	4923.5 ± 40.5 &

As revealed by Student's t-test, (1) ultrashort wave treatment significantly reduced the total contracture and the myogenic contracture compared with self-recovery ($P < 0.05$). (2) ultrashort wave treatment significantly increased the CSA compared with self-recovery ($P < 0.05$). * $P < 0.05$ that versus the Group R-1, # $P < 0.05$ that versus the Group R-2, & $P < 0.05$ that versus the Group R-4.

Figures

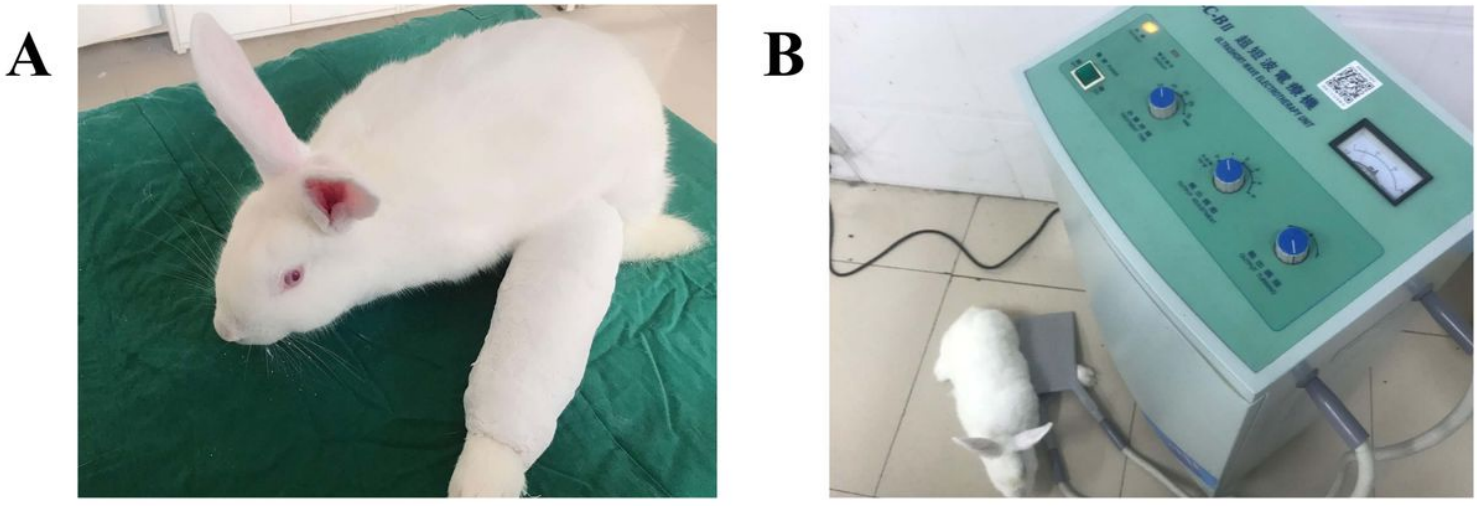

Figure 1

(A) The rabbits underwent unilateral immobilization of a knee joint at full extension using a plaster cast from groin to proximal toes. (B) The rabbits underwent the ultrashort wave treatment.

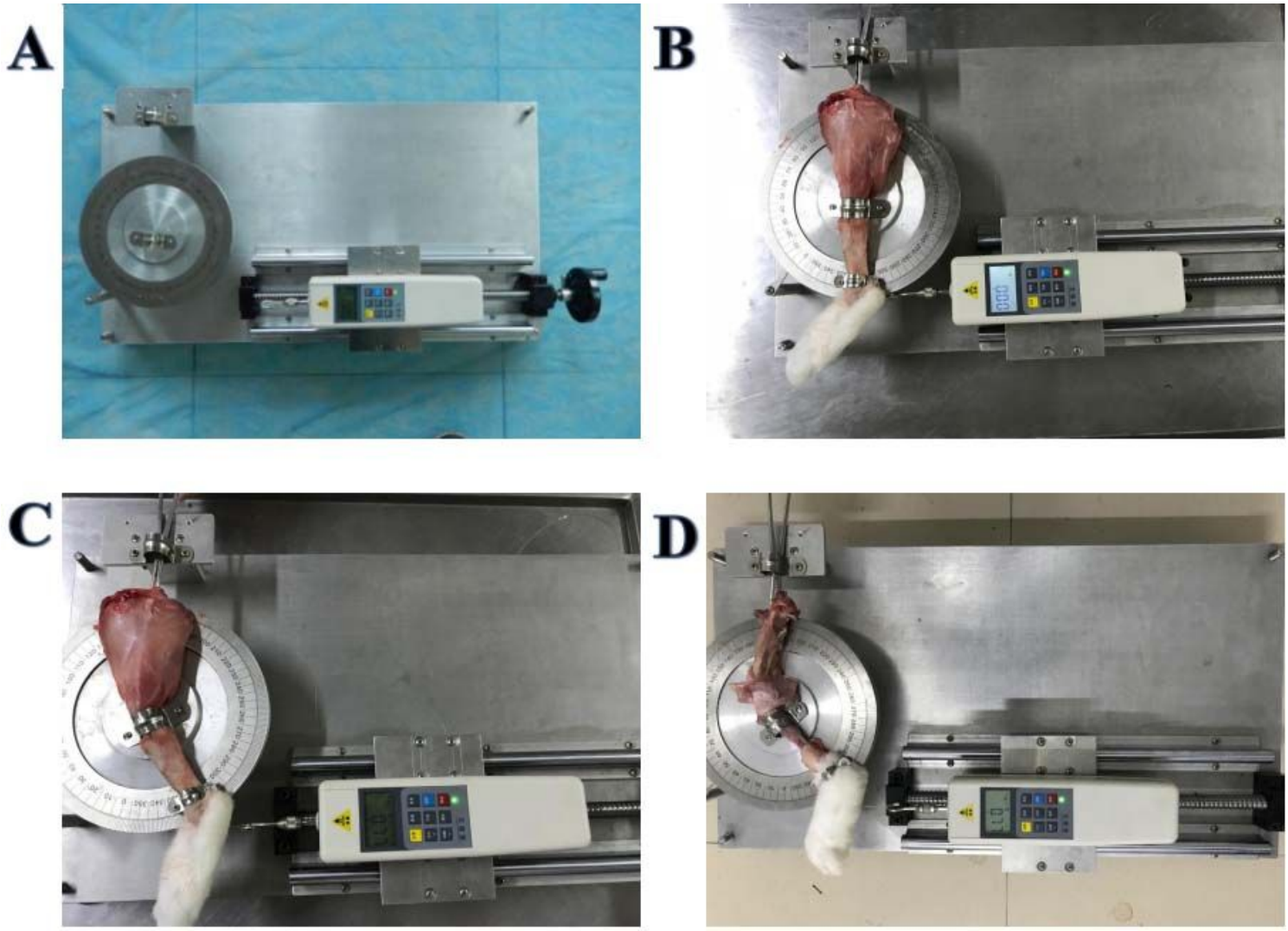

Figure 2

(A) The joint motion measuring instrument used is shown in the figure. (B) (C, D) The left hind limb of the rabbit was fixed to the joint motion measuring instrument. At this time, the display on the screen of the digital dynamometer showed a force of 0. Then the handle was turned and always maintained a torque of 0.077 N.m, and the left knee joint ROM before and after myotomy was obtained by the scale of the dial.

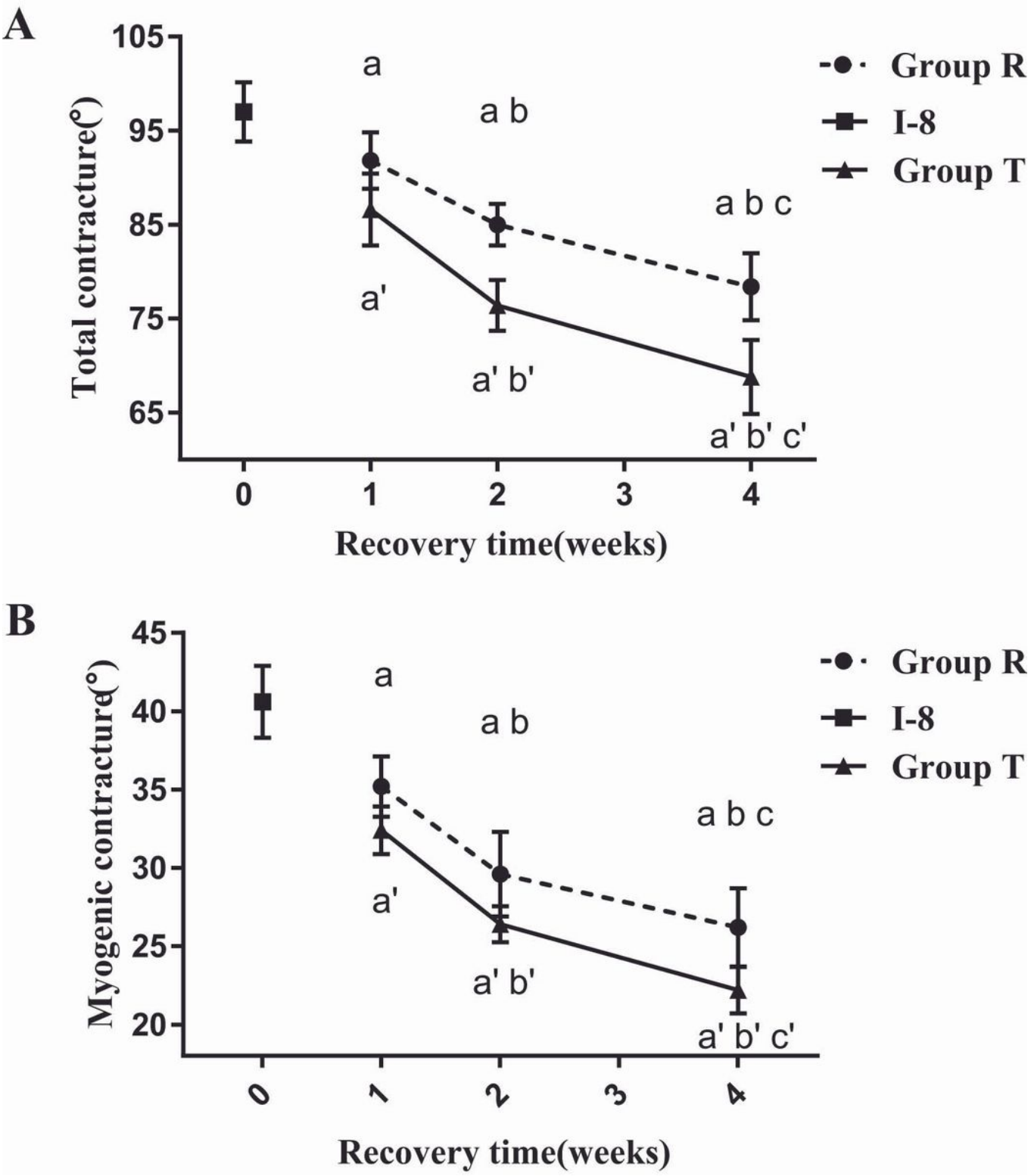

Figure 3

Effects of self-recovery and ultrashort wave treatment on the total and the myogenic contracture. a $P < 0.05$ that versus the Group I-8, b $P < 0.05$ that versus the Group R-1, c $P < 0.05$ that versus the Group R-2, a' $P < 0.05$ that versus the Group I-8, b' $P < 0.05$ that versus the Group T-1, c' $P < 0.05$ that versus the Group T-2.

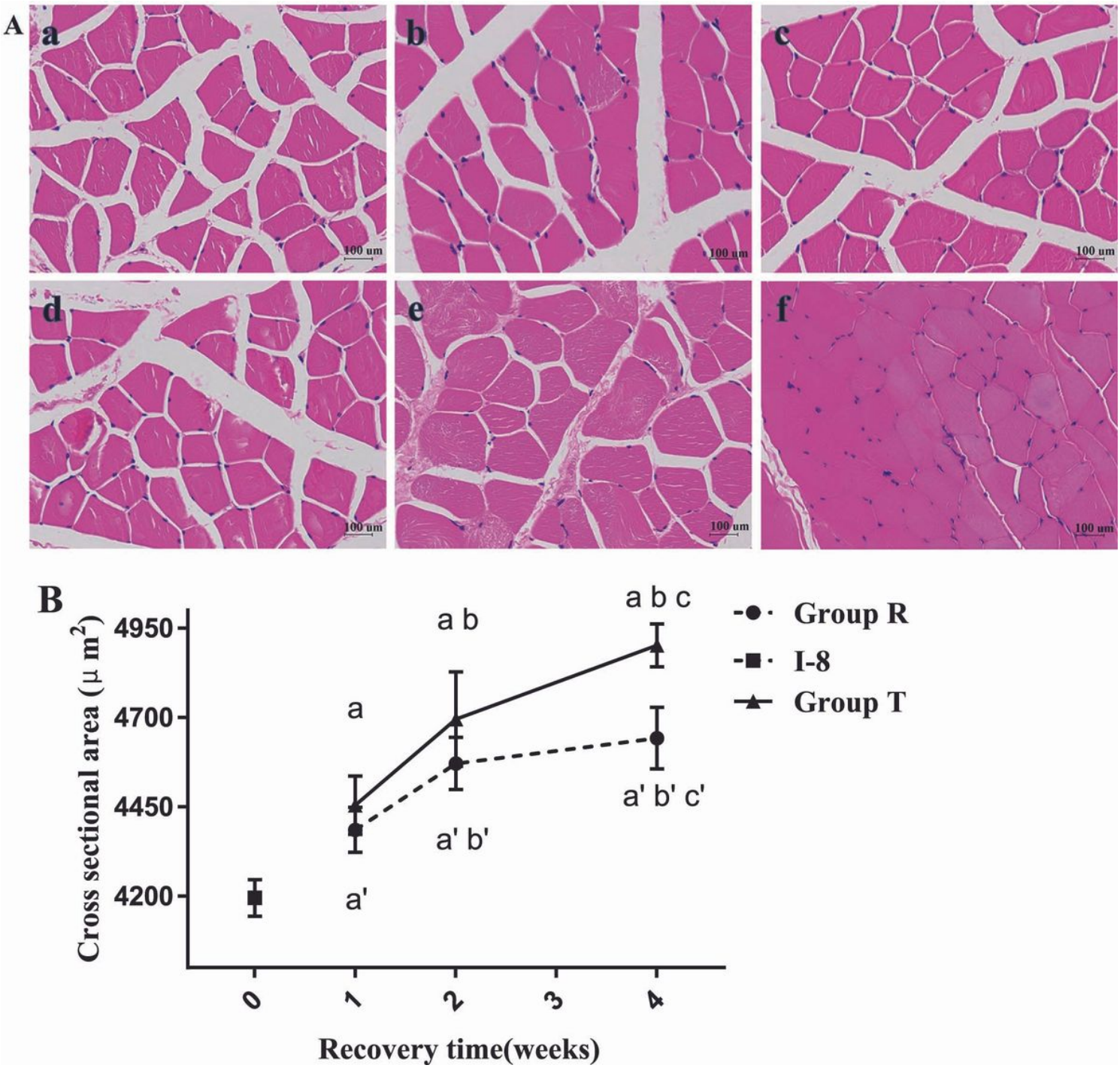

Figure 4

(A) Morphological changes of the rectus femoris. From a to c were shown as the cross sections of the rectus femoris fibers after 1, 2, 4 weeks of self-recovery respectively, and from d to f were shown as the cross sections of the rectus femoris fibers after 1, 2, 4 weeks of ultrashort wave therapy respectively Scale bar = 100 μm. (B) Effects of self-recovery and ultrashort wave treatment on the CSA. a $P < 0.05$ that versus the Group I-8, b $P < 0.05$ that versus the Group R-1, c $P < 0.05$ that versus the Group R-2, a' $P < 0.05$ that versus the Group I-8, b' $P < 0.05$ that versus the Group T-1, c' $P < 0.05$ that versus the Group T-2.

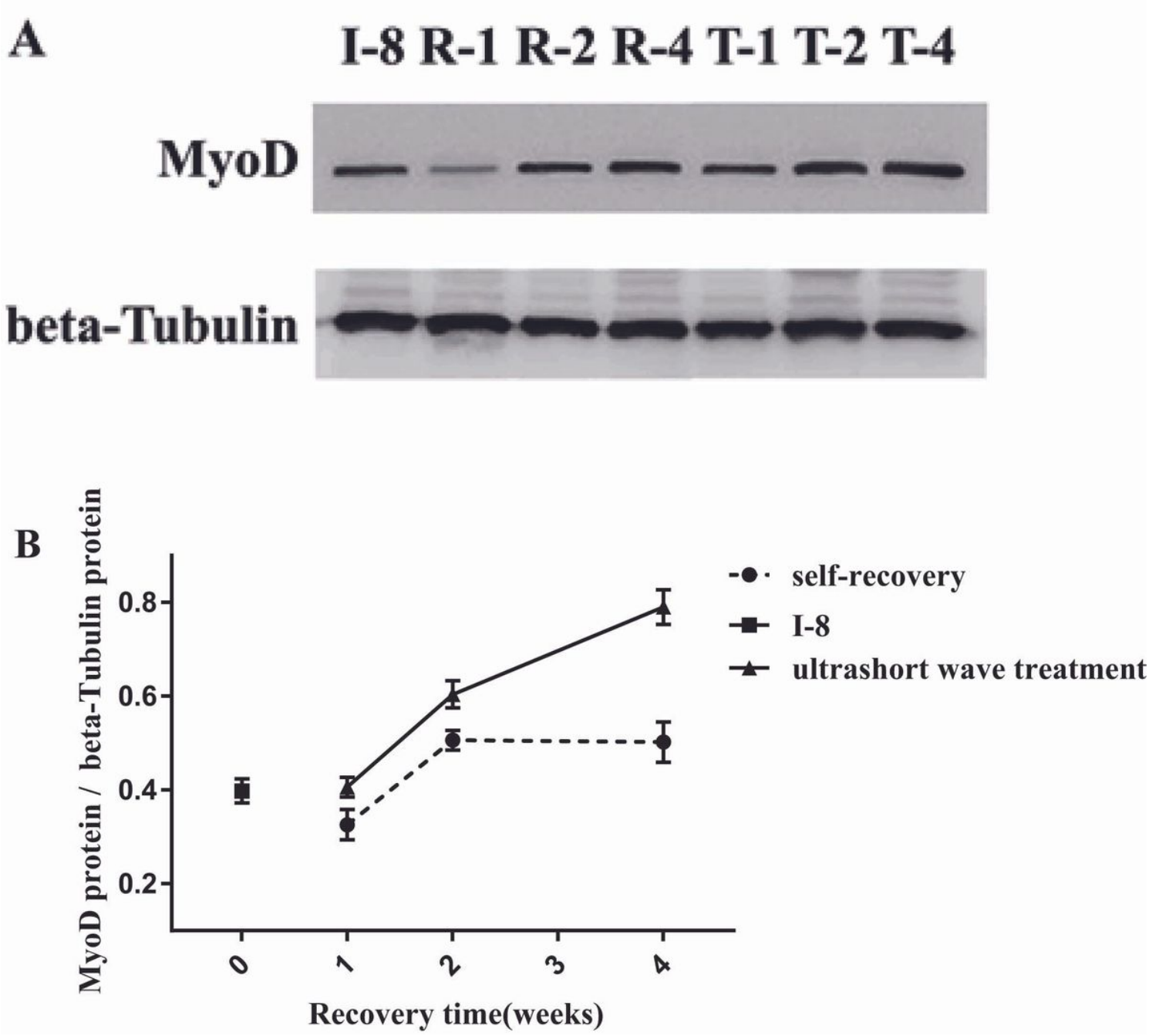

Figure 5

(A) The MyoD of the rectus femoris in each group. (B) Effects of self-recovery and ultrashort wave treatment on the average protein levels for MyoD. a $P < 0.05$ that versus the Group I-8, b $P < 0.05$ that versus the Group R-1, c $P < 0.05$ that versus the Group R-2, a' $P < 0.05$ that versus the Group I-8, b' $P < 0.05$ that versus the Group T-1, * $P < 0.05$ that versus the Group R-1, & $P < 0.05$ that versus the Group R-2, # $P < 0.05$ that versus the Group R-4.

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