

# Low-frequency Electrical Stimulation Alleviates Immobilization-evoked Disuse Muscle Atrophy via Repressing Autophagy in Skeletal Muscle of Rabbits

**A-Ying Liu**

The Second Hospital of Anhui Medical University

**Quan-Bing Zhang**

The Second Hospital of Anhui Medical University

**Hua-Long Zhu**

Anhui Medical University

**Yong-Wei Xiong**

Anhui Medical University

**Feng Wang**

The Second Hospital of Anhui Medical University

**Peng-Peng Huang**

The Second Hospital of Anhui Medical University

**Qi-Yu Xu**

The Second Hospital of Anhui Medical University

**Hua-Zhang Zhong**

The Second Hospital of Anhui Medical University

**Hua Wang**

Anhui Medical University

**Yun Zhou** (✉ [zhouyunanhui@sina.com](mailto:zhouyunanhui@sina.com))

The Second Hospital of Anhui Medical University

---

## Research Article

**Keywords:** disuse muscle atrophy, low-frequency electrical stimulation, autophagy, knee extension contracture

**Posted Date:** November 1st, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-1008815/v1>

**License:**   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

# Abstract

**Objective:** The present study was to investigate the effect of low-frequency electrical stimulation on disuse muscle atrophy and its mechanism in a rabbit model of extending knee joint contracture.

**Methods:** This study designed two experiments. In the time-point experiment, 24 rabbits were randomly divided into Control 1 (Ctrl1), immobilization for 2 weeks (I-2), I-4, and I-6 groups. In the intervention experiment, 24 rabbits were also randomly divided into Control 2 (Ctrl2), electrical stimulation (ES), natural recovery (NR) and electrical stimulation treatment (EST) groups. All intervention effects were assessed by evaluating the knee joint range of motion (ROM), cross-sectional area (CSA) of muscle and the expression of autophagy-related proteins.

**Results:** Time-point experiment showed that immobilization reduced knee ROM, muscle CSA, and activated autophagy in skeletal muscle. Levels of four autophagic proteins including p-mTOR, Atg7, p62 and LC3B-II, were significantly elevated in the skeletal muscle of I-4 group. The intervention experiment further presented that LFES significantly improved the immobilization-induced ROM and CSA reduction. Additionally, LFES significantly reversed autophagy activation of skeletal muscle caused by immobilization.

**Conclusions:** Low-frequency electrical stimulation alleviates immobilization-evoked disuse muscle atrophy maybe via inhibiting autophagy in skeletal muscle of rabbits.

## 1. Introduction

Trauma, joint fixation, nervous system damage, and prolonged bed rest may cause joint contractures, and long-term joint immobilization is a key factor in the formation of joint contractures [1–4]. As the largest and most complex joint of the human body, knee joint is the most important walking and weight-bearing joint of the lower limbs. Knee joint is easily injured due to severe trauma, imbalanced weight bearing, improper activity or excessive bear load [5]. Injured knee joints often need to be fixed [6], but prolonged inactivity can cause skeletal muscle atrophy and weakness. Studies have found that the etiology of joint contractures can be divided into two parts, such as myogenic component and articular component. In the early stage of joint contracture, myogenic factors are reversible in the natural recovery process, and the development of joint contracture to the stable stage is mainly caused by irreversible articular component [7]. Myogenic contracture is mainly manifested as disuse muscle atrophy caused by immobilization, and the cross-sectional area (CSA) of skeletal muscle fibers is significantly reduced [8–10]. Disuse muscle atrophy is considered to be an important part of myogenic contracture, which plays a role in promoting the occurrence and development of joint contracture [3]. The progression of skeletal muscle atrophy reduces physical activity, leading to bedridden. Therefore, preventing disuse muscle atrophy is very important to improve the quality of life.

Our previous studies have found that the early treatment of disuse muscle atrophy is beneficial to the rehabilitation of knee joint contracture [11]. Disuse muscle atrophy occurs due to accelerated proteolysis

or decreased synthesis, and proteolysis plays a leading role in certain types of atrophy caused by inactivity. Although all major proteolytic systems are involved in immobilization-triggered proteolysis in skeletal muscle, protein degradation induced by the autophagy-lysosome pathway plays a key role in muscle atrophy [12, 13]. Specht et al. [14] found that suspending the hind limbs of mice and fixing them with casts could aggravate muscle atrophy by stimulating autophagy. In human skeletal muscle, the mRNA expression of five autophagy-related genes (p62, LC3B, BECLIN-1, ATG12, and BNIP3) increased during the use of Donjoy splint fixation, and returned to the baseline levels during device trainings [15]. Correspondingly, studies have shown that muscle size can be maintained by repressing autophagy. Blocking the autophagy pathway with siRNA or chloroquine (CQ) could inhibit TGF- $\beta$ 1-mediated skeletal muscle atrophy [16]. However, in the field of rehabilitation medicine, whether physical factor therapy could reduce disuse muscle atrophy by inhibiting autophagy is still unclear.

Electrical stimulation (ES) is a safe and effective physical factor therapy [17]. Under certain conditions, ES could enhance the contractile function of muscle fibers and prevent skeletal muscle atrophy. Low-frequency electrical stimulation (LFES) is used to treat disuse skeletal muscle atrophy caused by tetrodotoxin paralysis in rats. The results showed that LFES of 2 pulses per second is more effective than high-frequency electrical stimulation [18]. In the chronic-kidney-disease-induced skeletal muscle atrophy model, LFES improved protein metabolism and promoted skeletal muscle regeneration by up-regulating the IGF-1 signaling pathway [19]. LFES can improve disuse skeletal muscle atrophy, but there is no report about the treatment of disuse muscle atrophy in joint contracture models with LFES. As above, we hypothesize that LFES may improve disuse muscle atrophy by inhibiting immobilization-induced skeletal muscle autophagy.

In this study, rabbits were used to establish the clinical common knee joint extension contracture model [20]. In this study, we first examined whether lower limb plaster external fixation could induce autophagy in rabbit skeletal muscle in a time-dependent manner. Subsequently, we designed an intervention experiment to explore the role and mechanism of skeletal muscle autophagy in LFES improving disuse muscle atrophy.

## **2. Methods**

### **2.1 Animals and experimental materials**

Our procedures on rabbits obeyed the guidelines for humane treatment, which were set by Animal Ethics Committee of Anhui Medical University (LLSC20190761). A total number of 48 male skeletally mature New Zealand white rabbits (aged 3-4 months; weight 2-2.5 kg) were purchased from the Experimental Animal Center of Anhui Medical University. The rabbits were individually reared in a cage of 60×50×40 cm<sup>3</sup>, with an ambient temperature of 24°C, and a light-dark cycle for 12 hours. Rabbits had unlimited activities in cages with enough food and water. All rabbits were fed with a standard rabbit diet for two weeks before the experiment.

Electronic Acupuncture Treatment Instrument (SDZ-Ⅱ type) was purchased from Hwato Co (Suzhou, China). The joint range of motion measuring instrument (ZL201720251124.6) with utility model patent was designed by our research group. Antibodies against Atg7(ab133528), LC3B-I/II (ab243506) and p62 (ab56416) were bought from Abcam (Cambridge, MA). p-mTOR (5536S) was bought from Cell Signaling Technology (Beverly, MA). GAPDH (F2612) antibody was bought from Santa Cruz Biotechnologies. Thiazolyl Blue Tetrazolium Bromide (MTT, M8180) was purchased from Solarbio (Beijing, China). Chemiluminescence (ECL) detection kit was from Thermo Scientific (Massachusetts, US).

## 2.2 Grouping and intervention measures

The whole experiment was divided into two parts. In the first part of the experiment, to explore the effects of immobilization on skeletal muscle autophagy, disuse muscle atrophy and joint contracture, 24 rabbits were randomly divided into control 1 group (Ctrl1 group), immobilization for 2 weeks group (I-2 group), immobilization for 4 weeks group (I-4 group) and immobilization for 6 weeks group (I-6 group), each group has 6 animals. Three groups of rabbits in need of immobilization were anesthetized by injection of 30 mg/kg sodium pentobarbital through ear vein and the left knee joints were fixed in extension. In the Ctrl1 group, the rabbits moved freely for 6 weeks; in the I-2, I-4, and I-6 groups, plaster casts were used to immobilize the rabbit knee joint from the groin to the proximal interphalangeal joint at full extension posture [20], removing the tubular plaster at the end of each fixed time point.

In the second part of the experiment, in order to study the therapeutic effect and mechanism of low-frequency electrical stimulation on disuse muscle atrophy and joint function, 24 rabbits were randomly divided into control 2 group (Ctrl2 group), natural recovery group (NR group), electrical stimulation treatment group (EST group) and pure electrical stimulation group (ES group), with 6 rabbits in each group. (A table with group characteristics is in the Supplementary Table S1 section.) In the Ctrl2 group, the rabbits were free to move for 7 weeks; in the ES group, the rabbits were free to move for 4 weeks, followed by 10 Hz low-frequency electrical stimulation treatment for 3 weeks; in the NR group, the rabbits' left knee joint were fixed as described above, the plaster was removed after 4 weeks of immobilization, followed by natural recovery for 3 weeks; in the EST group, the left knee joint of the rabbit was fixed for 4 weeks and then the plaster was removed, followed by low-frequency electrical stimulation at 10 Hz for 20 min a day for 3 weeks.

## 2.3 Low frequency electrical stimulation treatment

Each rabbit in the ES and EST groups received 3 weeks of 10Hz low-frequency electrical stimulation once a day for 20 minutes with an Electronic Acupuncture Treatment Instrument (SDZ-Ⅱ). The intervention site of electrical stimulation was the quadriceps femoris of the left hind limb of the rabbit. First, the hair of the left hind leg was shaved off, and then two 3 × 3 cm<sup>2</sup> non-woven silica gel electrode sheets were attached to the skin on the front side of the left hind leg. The distance between the two electrodes was 0.5 cm. The output current of electronic acupuncture instrument was less than 10mA. We adjust the size of the output current to cause the quadriceps muscle contraction, but not to cause the rabbit's strong resistance. A current of 5 mA could cause obvious muscle contraction without the rabbit's excessive struggling in our

preliminary experiment, so the current was set to 5 mA in our former experiment. The electronic acupuncture instrument worked in an intermittent wave mode with a pulse duration of 15 sec and a pause time of 5 sec.

## **2.4 Tissue preparation and joint ROM measurement**

Each rabbit's left hind limb was dislocated at the left hip joint after euthanasia with an overdose of sodium pentobarbital via an auricular vein, this method of euthanasia was approved by Animal Ethics Committee of Anhui Medical University (LLSC20190761). The starting point of the thigh muscles at the hip joint was cut off, and the left hind limb was completely detached from the torso. As in previous experiment, a joint range of motion measuring instrument was used to measure the range of motion of the left knee joint [20]. The proximal end of femur, the proximal end and the distal end of tibia were fixed on the arthrometer with a metal clamp. All knee joints started at 0° flexion position before applying the force. The driving wheel rotated to drive the dial to rotate, and the tibia rotated indirectly, and the femur remained motionless. Since the radius of the dial is fixed, the torque applied can be calculated by multiplying the force with the dial constant radius. In our previous experiment, by measuring the range of motion of the normal rabbits, it was found that the torque of 0.077 N.m could pull the knee joint of rabbits to about 140 degrees of buckling. After this, even though the torque continued to increase the bending angle of knee joint was difficult to increase. Therefore, we used 0.077 N.m as the standard torque to measure the knee joint ROM. The grouping information of rabbits was kept secret from the surveyors. The ROM measurements were made by 2 surveyors and repeated 3 times for each rabbit. The surveyors kept their measurements secret from each other and the buckling angle of each rabbit was the average of six measurements. (A table with contracture angle is in the Supplementary Table S2 section.)

Subsequently, three muscle tissue specimens were removed from the middle of the separated rectus femoris muscle, about  $1 \times 1 \times 0.5 \text{ cm}^3$  in size, two samples were used for hematoxylin-eosin and immunofluorescence staining, and the other sample was stored in a refrigerator at  $-80^\circ\text{C}$  for the detection of skeletal muscle autophagy proteins.

## **2.5 Hematoxylin and eosin (H&E) staining**

Rabbit rectus femoris tissues were fixed with 4% paraformaldehyde (PFA) and then embedded in paraffin. The rectus femoris sections were stained using hematoxylin and eosin (H&E) staining. In the case of magnification of 400 times, the cross section of the rectus femoris muscle was photographed with Nikon TE2000-U microscope (Nikon Corporation, Tokyo, Japan), and 4 fields were randomly selected for each HE staining section. Image-Pro Plus 6.0 software was used to count the number of muscle fibers and the total area of muscle fibers in each field. The average muscle fiber area under each field was statistically analyzed with SPSS 23.0. (A table with total number of myofiber cells is in the Supplementary Table S2 section.)

## **2.6 Western blotting**

Weighed the rectus femoris muscle approximately 70-80 mg and added 600µl lysis buffer to each sample which was a 100:1 proportion of RIPA and PMSF. The homogenized tissue sample was transferred to a 1.5 ml EP tube, and the EP tube was put into a centrifuge (4°C). The tube was centrifuged at 12,000 g of centrifugal force for 15min. After centrifugation, 300µl of the intermediate layer clear liquid was absorbed to prepare for the subsequent protein quantification of BCA method. The antibody diluent we used was 5% skimmed milk (5g powdered milk plus 100ml TBST). The loading volume of the sample is determined according to the expression intensity of the protein. For the reference protein with relatively stable expression, such as GAPDH, the loading volume of 8µg is sufficient; however, for the target protein with relatively weak expression, such as LC3B, the loading volume usually needs to reach 75µg. For other target proteins, the loading volume of the sample is determined according to the expression intensity of the protein. Total lysate was separated with 12.5% SDS-PAGE electrophoresis buffer and then transferred onto PVDF membranes. The membranes were first sealed with milk for 1.5h, and then incubated with primary antibodies for 1-2h. GAPDH was applied as a loading control. After being washed, the membranes were incubated with secondary antibodies which were conjugated to HRP for 90 minutes. After being washed, the enhanced chemiluminescence reagent was used for development. The signal was then detected with a digital imaging equipment.

## 2.7 Immunofluorescence

Thin sections (10 µm) of rectus femoris were fixed for 1 h with 4% PFA. Nonspecific binding sites in the slides were blocked using 10% normal goat serum. The slides were incubated for 2 hours with LC3B (1:200) at 37°C. The slides were incubated by the Alexa Fluor 488 conjugated secondary antibody (711-545-152, Jackson Immuno Research) for 90 min after PBS washing. The sections were stained with DAPI (C1002, Beyotime) for 5 min. All sections were then mounted and observed using a fluorescence microscope (BX53F, Olympus) under a 400x magnification field. Four fields were randomly selected and photographed for each slice. LC3B positive points in each visual field were counted out for statistical analysis. The mean value of green fluorescence points in individual muscle fiber under each field of vision was calculated.

## 2.8 Statistical analysis

Mean ± SD was used to present the quantified data. All data were entered and analyzed in SPSS (version 23.0). Mean differences among groups of rabbits in (1) range of motion, (2) cross-sectional area of the RF, and (3) expression of p-mTOR, Atg7, LC3B-I/II and p62 proteins associated with muscle autophagy, were assessed using one-way analysis of variance. When analysis of variance detected differences, Bonferroni or Tamhane's T2 tests were used to assess multiple comparisons between groups. To examine all pairwise comparisons among groups of rabbits.  $P < 0.05$  indicated that the difference was statistically significant.

## 3. Results

### **3.1 Immobilization induces disuse muscle atrophy and joint contracture in rabbits.**

The knee joint flexion ROM of the 4 groups of rabbits is shown in Fig. 1A (Ctrl1 group:  $144.27 \pm 1.99^\circ$ , I-2 group:  $81.83 \pm 16.64^\circ$ , I-4 group:  $54.48 \pm 13.32^\circ$ , I-6 group:  $39.38 \pm 8.83^\circ$ ). By comparing rabbits with different fixed time, we found that rabbits with longer fixed time had more significant reduction in knee ROM. After 2 weeks of immobilization, knee flexion ROM of I-2 group was less than Ctrl1 group ( $P < 0.01$ ). The ROM of I-4 group is further reduced compared with I-2 group ( $P < 0.05$ ). There was a statistically difference in the ROM between I-4 group and Ctrl1 group ( $P < 0.01$ ). The ROM of rabbits in I-6 group was further reduced compared with I-4 group ( $P < 0.05$ ). There was a statistically difference in the ROM between I-6 group and Ctrl1 group ( $P < 0.01$ ). There was also a statistically difference in the ROM between I-6 group and I-2 group ( $P < 0.01$ ). As shown in Fig. 1B, the rectus femoris muscle disuse atrophy was more obvious in the group with long time immobilization. As presented in Fig. 1C, compared with the Ctrl1 group ( $2962.89 \pm 350.82 \mu\text{m}^2$ ), the I-6 group ( $1711.81 \pm 208.92 \mu\text{m}^2$ ) had the most obvious reduction in CSA value ( $P < 0.01$ ); there was a statistically difference in CSA between the I-2 group ( $2021.56 \pm 451.15 \mu\text{m}^2$ ) and the Ctrl1 group ( $P < 0.05$ ); the CSA of I-4 group ( $1871.49 \pm 737.48 \mu\text{m}^2$ ) compared with the Ctrl1 group was also statistically significant ( $P < 0.05$ ); there was no statistical difference in CSA between group I-2, I-4 and I-6 ( $P > 0.05$ ).

### **3.2 Immobilization induces the activation of rabbit skeletal muscle autophagy**

Rectus femoris was used to test the effect of immobilization on autophagy in skeletal muscle. As shown in Fig. 2A-B, immobilization increased the expression levels of p-mTOR, and Atg7 proteins in rabbit skeletal muscle. The protein expression levels of p-mTOR in the I-4 group was higher than Ctrl1 group ( $P < 0.05$ ). The protein expression level of Atg7 in I-2 group was higher than Ctrl1 group ( $P < 0.05$ ). The protein expression level of Atg7 in I-4 group was higher than Ctrl1 and I-2 groups ( $P < 0.01$ ). The protein expression level of Atg7 in I-6 group was lower than I-2 group ( $P < 0.05$ ) and I-4 group ( $P < 0.01$ ).

As shown in Fig. 2C-D, immobilization caused the increase of LC3B-II and p62 proteins level in rabbit skeletal muscle. Among them, the protein expression levels of LC3B-II and p62 in the I-4 group were higher than those of Ctrl1 group ( $P < 0.05$ ). As shown in Fig. 3A-B, LC3 immunofluorescence staining with frozen sections of rectus femoris provides further evidence for autophagy in atrophic skeletal muscle. The results showed that LC3 positive points in the I-4 group were more than those in Ctrl1 group, and the difference was statistically significant ( $P < 0.05$ ) (Fig. 3B).

### **3.3 Low-frequency electrical stimulation improves disuse muscle atrophy and knee joint contracture.**

The effect of low-frequency electrical stimulation on rabbit knee joint ROM is shown in Fig. 4A. Knee joint ROM in the NR group ( $60.67 \pm 5.71^\circ$ ) and EST group ( $84.27 \pm 5.66^\circ$ ) were lower than those in the Ctrl2

group ( $143.33 \pm 2.14^\circ$ ) and ES group ( $143.82 \pm 1.93^\circ$ ), the differences were statistically significant ( $P < 0.01$ ); the improvement of ROM in the EST group was greater than that of the NR group, and the difference was statistically significant ( $P < 0.01$ ). As shown in Fig. 4B, the improvement of skeletal muscle disuse atrophy in the EST group was greater than that in the NR group. The quantitative results in Fig. 4C showed that CSA of NR group ( $1628.99 \pm 486.12 \mu\text{m}^2$ ) was lower than those of the Ctrl2 group ( $2962.89 \pm 350.82 \mu\text{m}^2$ ) and ES group ( $2928.44 \pm 160.23 \mu\text{m}^2$ ), and the difference was statistically significant ( $P < 0.01$ ); CSA of EST group ( $2486.92 \pm 455.99 \mu\text{m}^2$ ) was improved compared with the NR group, and the difference was statistically significant ( $P < 0.05$ ).

### **3.4 Low-frequency electrical stimulation reverses immobilization-triggered activation of autophagy in rabbit rectus femoris.**

In order to study the role of autophagy inhibition in the treatment of knee joint contracture by low-frequency electrical stimulation, the expression levels of p-mTOR and Atg7 proteins in rectus femoris were detected. As shown in Fig. 5A-B, low-frequency electrical stimulation inhibited the expression of p-mTOR, and Atg7 proteins in rabbit skeletal muscle. The protein expression level of p-mTOR in NR group was higher than those in Ctrl2 and ES groups ( $P < 0.01$ ). The p-mTOR expression level in EST group was lower than NR group ( $P < 0.01$ ). The protein expression level of Atg7 in NR group was higher than Ctrl2 and ES groups ( $P < 0.01$ ). Atg7 protein expression level of EST group was lower than NR group ( $P < 0.05$ ).

To further explore the therapeutic effect of low frequency electrical stimulation on joint contracture, the expression levels of LC3B-II and p62 proteins in rectus femoris were also detected. As shown in Fig. 5C and D, the expression levels of LC3B-II and p62 in NR group were higher than those of the Ctrl2 and ES groups, and the differences were statistically significant ( $P < 0.01$ ); compared with NR group, the expressions of LC3B-II and p62 in EST group were inhibited, and the differences were statistically significant ( $P < 0.01$ ).

## **4. Discussion**

At present, the flexion-type knee joint contracture model has been used in the most experimental studies of knee joint contracture. When modeling, the animal's knee joint is fixed at about  $150^\circ$  in the flexion position, which leads to limited knee extension [21–24]. According to the internationally universal neutral-zero method, the neutral position of the knee joint is the extension position, which is calculated as  $0^\circ$ . The ROM of a normal knee joint is  $120^\circ \sim 150^\circ$  in flexion and  $5^\circ \sim 10^\circ$  in hyperextension. The functional position of the knee joint is  $15^\circ \sim 20^\circ$  of flexion. Knee joint injuries usually require fixation in an extended or functional position to promote tissue healing. Therefore, the extension position or functional position fixation is a common orthopedic treatment for knee trauma or other musculoskeletal diseases [25]. To be consistent with clinical practice, we used tubular plaster external fixation to establish a rabbit knee joint extension contracture model [20]. Due to individual differences and difficulty in cooperation of rabbits, the objectivity of knee joint ROM measurement in vivo is difficult to guarantee. Therefore, we separated the

left hind limb of the rabbit to measure the range of motion of the knee joint. The advantage of this method is that it is easier to obtain objective measurement results under standard torque [11, 20, 26]. As mentioned above, the extension type knee joint contracture model we designed is more suitable for the patient's clinical situation than other flexion type knee joint contracture models.

Skeletal muscles play an important role in the pivotal body functions such as breathing and movement [27–29]. One study found that skeletal muscle weight and cross-sectional area were significantly reduced in the suspension-induced disuse muscle atrophy model [30]. Time-point experiment found that in the formation of rabbit knee joint contracture caused by fixation, disuse atrophy of skeletal muscle appeared. Quantitative analysis of HE staining showed that skeletal muscle atrophy progressed rapidly in the 2 weeks of fixation, and progressed slowly after 2-6 weeks of fixation. Cessation of skeletal muscle use could lead to muscle atrophy, which is reversible after a short period of non-use [31]. Intervention experiment found that, the indexes of CSA and ROM in NR group did not return to normal level compared with Ctrl2 group, indicating that short-term natural recovery after 4 weeks of fixation could not completely reverse skeletal muscle atrophy and joint mobility limitation. Compared with NR group, the ROM and CSA of EST group were significantly improved. These results indicate that LFES has a certain therapeutic effect on improving joint function and disuse muscle atrophy.

Although all major proteolytic systems are involved in inactivity-induced proteolysis in skeletal muscle, there is an increasing evidence that the autophagy lysosomal pathway plays an important role [32, 33]. So far, no studies have investigated the effect of autophagolysosomal system on disuse muscle atrophy in the knee joint contracture model. In this study, we focused on the effect of skeletal muscle autophagy on early disuse muscle atrophy in the model of rabbit knee joint contracture. p-mTOR and Atg7 are important upstream regulators of autophagy [34, 35]. During the formation of autophagosomes, LC3-II specifically binds to autophagosomes or autophagolysosomes, and the increased expression of LC3-II is a signal of autophagy activation [36–38]. As one of the most important substrates of autophagy, p62 is a vital receptor in autophagy and is generally regarded as an indicator of autophagy degradation [39, 40]. Our results showed that immobilization significantly increased the overexpression of four autophagy-specific protein markers p-mTOR, Atg7, LC3B-II and p62 in rabbit skeletal muscle. Among them, skeletal muscle autophagy reached the highest level after 4 weeks of fixation, and the autophagy trend decreased after 6 weeks of fixation. Although the autophagy of skeletal muscle was reduced in group I-6, the atrophy of skeletal muscle in group I-6 was the most obvious. The reason for the results may be related to the fixation time and the activation of other protein degradation pathways. In conclusion, plaster external fixation during the formation of joint contracture triggers disuse atrophy of skeletal muscle via activating the autophagy pathway mediated by p-mTOR signal.

It is known that LFES could improve muscle atrophy to a certain extent [41–43] Since autophagy of skeletal muscle in I-4 group increased the most, we performed LFES on the quadriceps muscle after 4 weeks of fixation in the intervention experiment. In addition, the results of further intervention experiment show that the expression of autophagy related protein in EST group was significantly lower than that in NR group. Skeletal muscle CSA and knee ROM in EST group were significantly improved compared with

NR group. Due to the limitation of experimental conditions, this study only used LFES to treat disuse muscle atrophy caused by knee joint immobilization. The results showed that disuse muscle atrophy and joint contracture caused by immobilization were not completely reversed. It is necessary to combine with other rehabilitation methods to explore a comprehensive treatment plan for disuse muscle atrophy and knee joint contracture.

## 5. Conclusion

Short exposure to immobilization could induce disuse muscle atrophy and skeletal muscle autophagy in the rabbit model of extension knee joint contracture. Low-frequency electrical stimulation protects against immobilization-induced disuse muscle atrophy, which was associated with autophagy inhibition in skeletal muscle of rabbits. These data provide the experimental basis for the earlier prevention and treatment of disuse muscle atrophy caused by immobilization.

## Abbreviations

Ctrl1: Control 1 group; I-2: immobilization for 2 weeks group; I-4: immobilization for 4 weeks group; I-6: immobilization for 6 weeks group; Ctrl2: Control 2 group; ES: electrical stimulation group; NR: natural recovery group; EST: electrical stimulation treatment group; ROM: range of motion; CSA: cross-sectional area; LFES: low-frequency electrical stimulation; p-mTOR: phosphorylated-mechanistic target of rapamycin; Atg7: autophagy related gene 7; LC3: microtubule-associated protein 1 light chain 3; p62: sequestosome 1 protein; GAPDH: glyceraldehyde phosphate dehydrogenase.

## Declarations

## Acknowledgements

The content is solely the responsibility of the authors. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## Authors' contributions

A-Ying Liu and Quan-Bing Zhang conceived of the study and participated in its design and drafted the manuscript. Hua-Long Zhu and Yong-Wei Xiong participated in the design of the study and performed the statistical analysis. Feng Wang and Peng-Peng Huang carried out the molecular studies. Qi-Yu Xu and Hua-Zhang Zhong carried out the animal experiments. Hua Wang and Yun Zhou participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

## Funding

This work was supported by Clinical Medicine Discipline Construction Project of Anhui Medical University in 2021 (2021LCXK031), Anhui Key Research and Development Program (201904a07020067) and Anhui Medical University Research Foundation (2018xkj050).

## Availability of data and material

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

## Ethics approval and consent to participate

Animal care and experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Anhui Medical University and were approved by the Institutional Animal Care and Use Committee (LLSC20190761).

## Consent for publication

Not applicable.

## Competing interests

The submitted content has not been previously published and is not currently under consideration for publication in any other scientific journal. We have no conflicts of interest to disclose.

## Author details

<sup>1</sup>Department of Rehabilitation Medicine, The Second Hospital of Anhui Medical University, Hefei, China. <sup>2</sup>Key Laboratory of Environmental Toxicology of Anhui Higher Education Institutes, Hefei, China. <sup>3</sup>Department of Toxicology, School of Public Health, Anhui Medical University, Hefei, China. <sup>4</sup>Department of Orthopedics, The Second Hospital of Anhui Medical University, Hefei, China.

## References

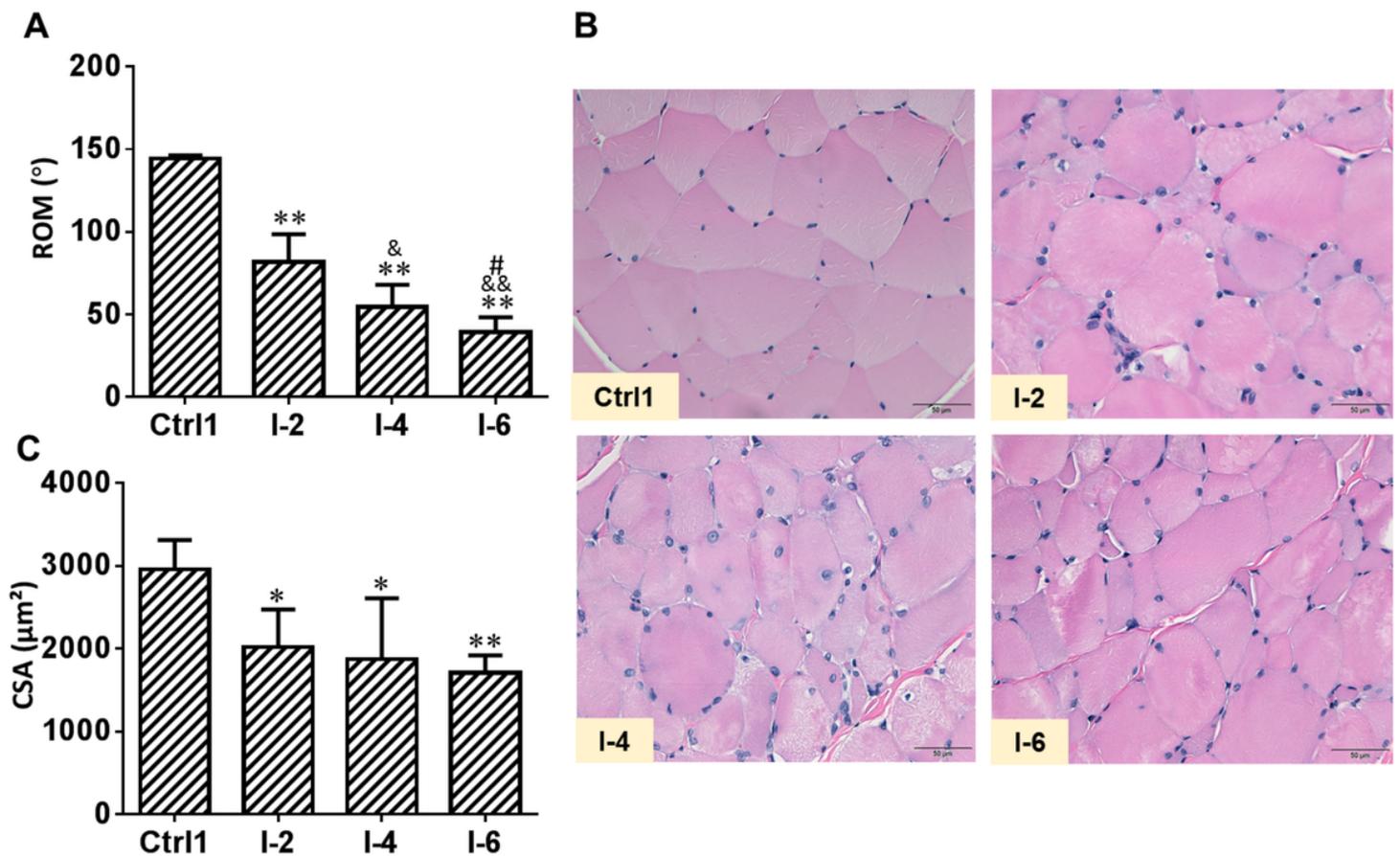
1. Baranowski A, Schlemmer L, Förster K, et al. Effects of losartan and atorvastatin on the development of early posttraumatic joint stiffness in a rat model. *Drug Des Devel Ther.* 2019; 13: 2603–2618.

2. Sotobayashi D, Kawahata H, Anada N, et al. Therapeutic effect of intra-articular injection of ribbon-type decoy oligonucleotides for hypoxia inducible factor-1 on joint contracture in an immobilized knee animal model. *J Gene Med*. 2016; 18(8): 180–192.
3. Wang F, Li W, Zhou Y, et al. Radial extracorporeal shock wave reduces myogenic contracture and muscle atrophy via inhibiting NF- $\kappa$ B/HIF-1 $\alpha$  signaling pathway in rabbit. *Connect Tissue Res*. 2021; 20(5): 1–10.
4. Wong K, Trudel G, Laneuville O. Intra-articular collagenase injection increases range of motion in a rat knee flexion contracture model. *Drug Des Devel Ther*. 2017; 12: 15–24.
5. Stiefel EC, Mcintyre L. Arthroscopic Lysis of Adhesions for Treatment of Post-traumatic Arthrofibrosis of the Knee Joint. *Arthrosc Tech*. 2017; 6(4): 939–944.
6. Clavet H, Hebert PC, Fergusson D, et al. Joint contracture following prolonged stay in the intensive care unit. *Can Med Assoc J*. 2008; 178(6): 691–697.
7. Trudel G, Laneuville O, Coletta E, et al. Quantitative and temporal differential recovery of articular and muscular limitations of knee joint contractures; results in a rat model. *J Appl Physiol*. 2014; 117(7): 730–737.
8. Chimoto E, Hagiwara Y, Ando A, et al. Progression of an arthrogenic motion restriction after immobilization in a rat experimental knee model. *Upsala J Med Sci*. 2007; 112(3): 347–355.
9. Aihara M, Hirose N, Katsuta W, et al. A new model of skeletal muscle atrophy induced by immobilization using a hook-and-loop fastener in mice. *J Phys Ther Sci*. 2017; 29(10): 1779–1783.
10. Kaneguchi A, Ozawa J, Minamimoto K, et al. Morphological and biomechanical adaptations of skeletal muscle in the recovery phase after immobilization in a rat. *Clin Biomech (Bristol, Avon)*. 2020; 75: 104992.
11. Wang F, Zhang QB, Zhou Y, et al. Effect of ultrashort wave treatment on joint dysfunction and muscle atrophy in a rabbit model of extending knee joint contracture: Enhanced expression of myogenic differentiation. *Knee*. 2020; 27(3): 795–802.
12. Cid-Díaz T, Santos-Zas I, González-Sánchez J, et al. Obestatin controls the ubiquitin-proteasome and autophagy-lysosome systems in glucocorticoid-induced muscle cell atrophy. *J Cachexia Sarcopenia Muscle*. 2017; 8(6): 974–990.
13. Yamada E, Bastie CC, Koga H, et al. Mouse skeletal muscle fiber-type-specific macroautophagy and muscle wasting are regulated by a Fyn/STAT3/Vps34 signaling pathway. *Cell Rep*. 2012; 1(5): 557–569.
14. Speacht TL, Krause AR, Steiner JL, et al. Combination of hindlimb suspension and immobilization by casting exaggerates sarcopenia by stimulating autophagy but does not worsen osteopenia. *Bone*. 2018; 110: 29–37.
15. Møller AB, Vendelbo MH, Schjerling P, et al. Immobilization decreases FOXO3a phosphorylation and increases autophagy-related gene and protein expression in human skeletal muscle. *Front Physiol*. 2019; 10: 736.

16. Yang X, Xue P, Liu X, et al. HMGB1/autophagy pathway mediates the atrophic effect of TGF- $\beta$ 1 in denervated skeletal muscle. *Cell Commun Signal*. 2018; 16(1): 97.
17. Gordon T, Sulaiman OA, Ladak A. Chapter 24: Electrical stimulation for improving nerve regeneration: where do we stand? *Int Rev Neurobiol*. 2009; 87: 433–444.
18. Dupont Salter AC, Richmond FJ, Loeb GE. Prevention of muscle disuse atrophy by low-frequency electrical stimulation in rats. *IEEE Trans Neural Syst Rehabil Eng*. 2003; 11(3): 218–226.
19. Hu L, Klein JD, Hassounah F, et al. Low-frequency electrical stimulation attenuates muscle atrophy in CKD—a potential treatment strategy. *J Am Soc Nephrol*. 2015; 26(3): 626–635.
20. Zhou Y, Zhang QB, Zhong HZ, et al. Rabbit model of extending knee joint contracture: Progression of joint motion restriction and subsequent joint capsule changes after immobilization. *J Knee Surg*. 2020; 33(1): 15–21.
21. Wong K, Trudel G, Laneuville O. Intra-articular collagenase injection increases range of motion in a rat knee flexion contracture model. *Drug Des Devel Ther*. 2017; 12: 15–24.
22. Yi X, Wang Z, Ren J, et al. Overexpression of chaperonin containing T-complex polypeptide subunit zeta 2 (CCT6b) suppresses the functions of active fibroblasts in a rat model of joint contracture. *J Orthop Surg Res*. 2019; 14 (1): 125.
23. Baranowski A, Schlemmer L, Förster K, et al. A novel rat model of stable posttraumatic joint stiffness of the knee. *J Orthop Surg Res*. 2018; 13(1): 185.
24. Zhang Y, Liu Z, Wang K, et al. Macrophage migration inhibitory factor regulates joint capsule fibrosis by promoting TGF- $\beta$ 1 production in fibroblasts. *Int J Bio Sci*. 2021; 17(7): 1837–1850.
25. Huang PP, Zhang QB, Zhou Y, et al. Effect of radial extracorporeal shock wave combined with ultrashort wave diathermy on fibrosis and contracture of muscle. *Am J Phys Med Rehabil*. 2020; 100(7): 643–650.
26. Zhang QB, Zhou Y, Zhong HZ, et al. Effect of stretching combined with ultrashort wave diathermy on joint function and its possible mechanism in a rabbit knee contracture model. *Am J Phys Med Rehabil*. 2018; 97(5): 357–363.
27. Dumont NA, Bentzinger CF, Sincennes MC, et al. Satellite Cells and Skeletal Muscle Regeneration. *Compr Physiol*. 2015; 5(3): 1027–1059.
28. Pedersen BK. Muscle as a secretory organ. *Compr Physiol*. 2013; 3(3): 1337–1362.
29. Langlois S, Cowan KN. Regulation of Skeletal Muscle Myoblast Differentiation and Proliferation by Pannexins. *Adv Exp Med Biol*. 2017; 925: 57–73.
30. Marzuca-Nassr GN, Kuwabara WMT, Vitzel KF, et al. Endoplasmic Reticulum Stress and Autophagy Markers in Soleus Muscle Disuse-Induced Atrophy of Rats Treated with Fish Oil. *Nutrients*. 2021; 13(7): 2298.
31. Wang X, Blagden C, Fan J, et al. Runx1 prevents wasting, myofibrillar disorganization, and autophagy of skeletal muscle. *Genes Dev*. 2005; 19(14): 1715–1722.

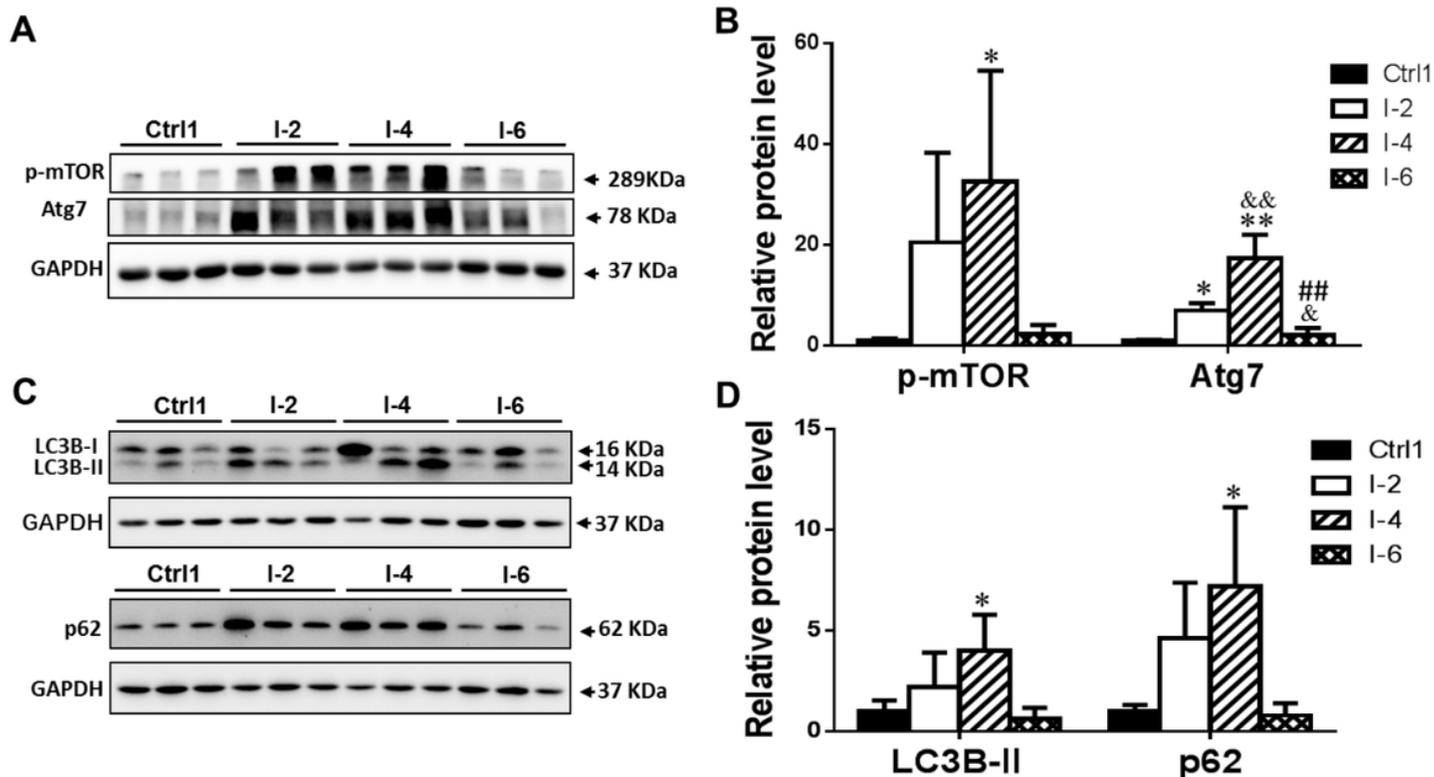
32. Powers SK, Ozdemir M, Hyatt H. Redox Control of Proteolysis During Inactivity-Induced Skeletal Muscle Atrophy. *Antioxid Redox Signal*. 2020; 33(8): 559–569.
33. Talbert EE, Smuder AJ, Min K, et al. Immobilization-induced activation of key proteolytic systems in skeletal muscles is prevented by a mitochondria-targeted antioxidant. *J Appl Physiol*. 2013; 115(4): 529–538.
34. Dunlop EA, Tee AR. mTOR and autophagy: a dynamic relationship governed by nutrients and energy. *Semin Cell Dev Biol*. 2014; 36: 121–129.
35. Zheng W, Xie W, Yin D, et al. ATG5 and ATG7 induced autophagy interplays with UPR via PERK signaling. *Cell Commun Signal*. 2019; 17(1): 42.
36. Ha SW, Weitzmann MN, Beck GR. Bioactive silica nanoparticles promote osteoblast differentiation through stimulation of autophagy and direct association with LC3 and p62. *Acs Nano*. 2014; 8(6): 5898–5910.
37. Huang R, Liu W. Identifying an essential role of nuclear LC3 for autophagy. *Autophagy*. 2015; 11(5): 852–853.
38. Tanida I, Minematsu-Ikeguchi N, Ueno T, et al. Lysosomal turnover, but not a cellular level, of endogenous LC3 is a marker for autophagy. *Autophagy*. 2005; 1(2): 84–91.
39. Sahani MH, Itakura E, Mizushima N. Expression of the autophagy substrate SQSTM1/p62 is restored during prolonged starvation depending on transcriptional upregulation and autophagy-derived amino acids. *Autophagy*. 2014; 10(3): 431–441.
40. Ichimura Y, Komatsu M. Selective degradation of p62 by autophagy. *Semin Immunopathol*. 2010; 32: 431–436.
41. Su Z, Hu L, Cheng J, et al. Acupuncture plus low-frequency electrical stimulation (Acu-LFES) attenuates denervation-induced muscle atrophy. *J Appl Physiol*. 2016; 120(4): 426–436.
42. Dupont Salter AC, Richmond FJ, Loeb GE. Prevention of muscle disuse atrophy by low-frequency electrical stimulation in rats. *IEEE Trans Neural Syst Rehabil Eng*. 2003; 11(3): 218–226.
43. Huang Y, Yu M, Kuma A, et al. Downregulation of let-7 by Electrical Acupuncture Increases Protein Synthesis in Mice. *Front Physiol*. 2021; 12: 697139.

## Figures



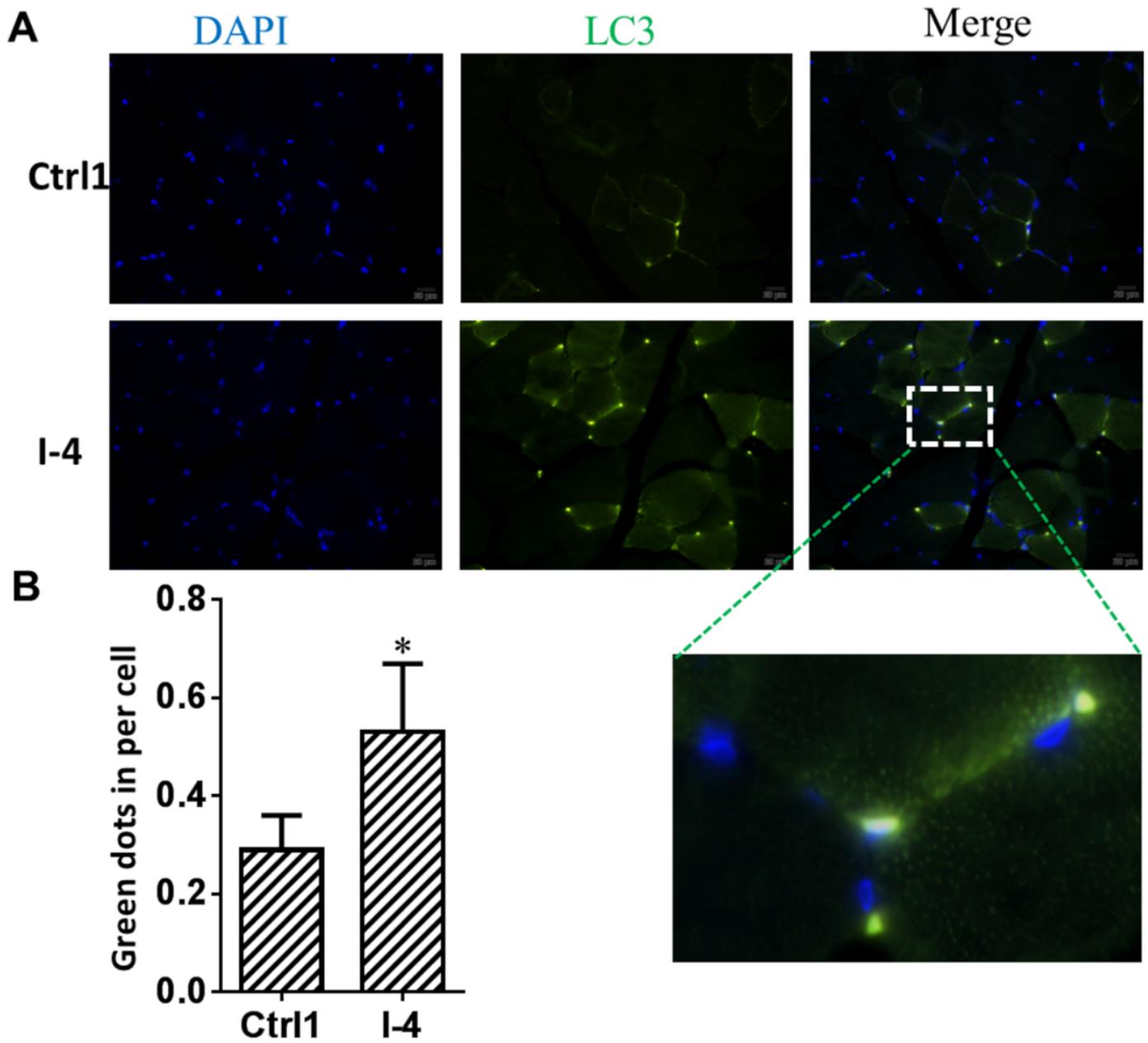
**Figure 1**

Immobilization induces disuse muscle atrophy and joint contracture in rabbits. Male rabbits were immobilized with plaster bandage on left hind knee joint in extension position. Rectus femoris was dissected at 2, 4 and 6 weeks after immobilization. (A) Knee joint range of motion (ROM). (B) Representative images from the cross section of skeletal muscle fibers using H&E staining. Scale bars represent 50 µm. (C) Quantitative analysis for cross-sectional area (CSA) of skeletal muscle fibers. Data were expressed as mean ± SD. n=6. Ctrl1: control 1 group; I-2: immobilization for 2 weeks group; I-4: immobilization for 4 weeks group; I-6: immobilization for 6 weeks group. \*P < 0.05, \*\*P < 0.01 compared to the Ctrl1; &P < 0.05, &&P < 0.01 compared to the I-2; #P < 0.05 compared to the I-4.



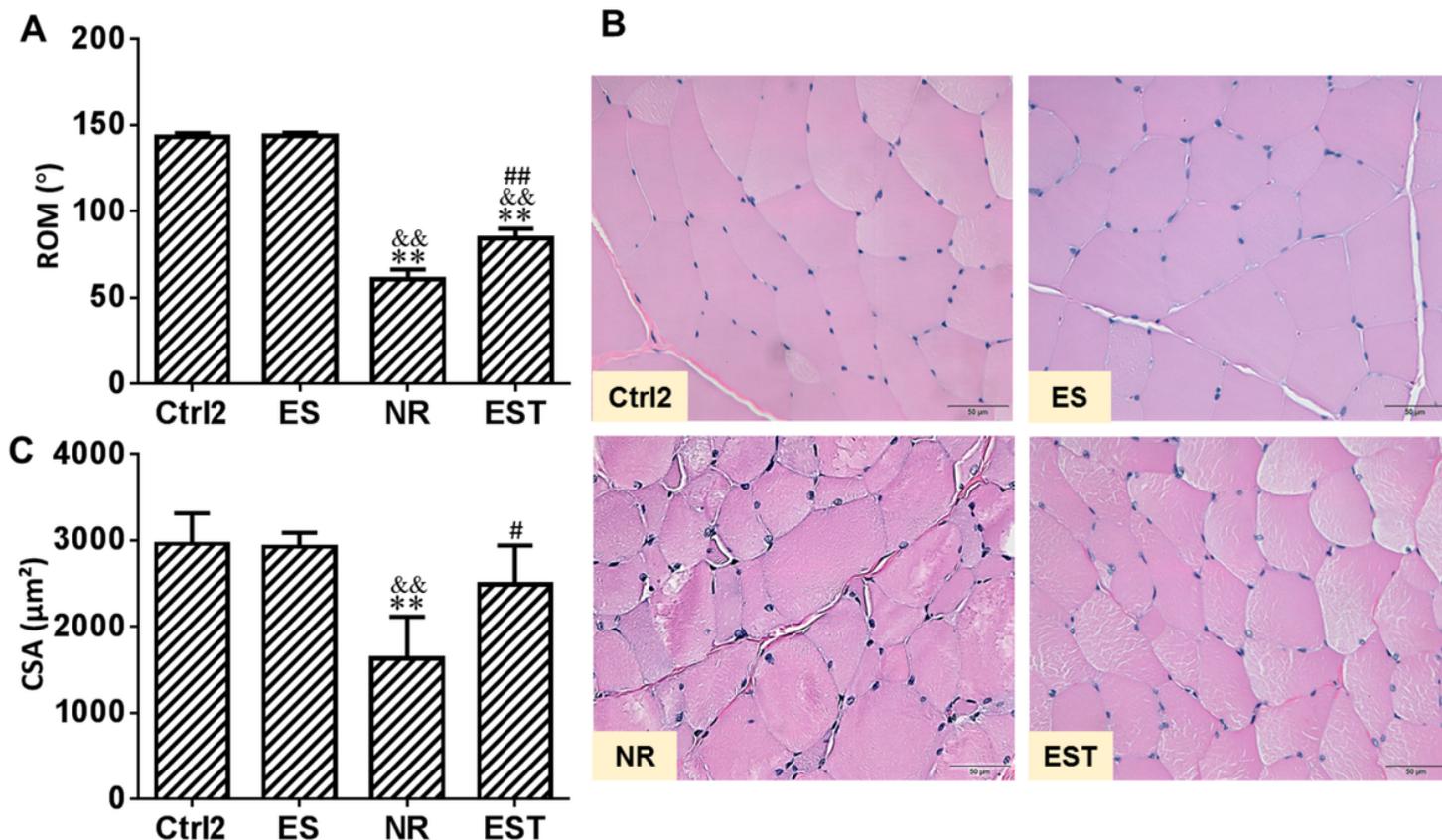
**Figure 2**

Immobilization induces activation of autophagy in rabbit skeletal muscle. Male rabbits were immobilized with plaster bandage on left hind knee joint in extension position. Rectus femoris was dissected at 2, 4 and 6 weeks after immobilization. (A) p-mTOR and Atg7 proteins were detected using western blotting. (B) Quantitative analysis for p-mTOR and Atg7. (C) LC3B-I/II and p62 proteins were detected using western blotting. (D) Quantitative analysis for LC3B-II and p62. Data were expressed as mean  $\pm$  SD. n=6. Ctrl1: control 1 group; I-2: immobilization for 2 weeks; I-4: immobilization for 4 weeks; I-6: immobilization for 6 weeks. \*P < 0.05, \*\*P < 0.01 compared to the Ctrl1; &P < 0.05, &&P < 0.01 compared to the I-2; ##P < 0.01 compared to the I-4.



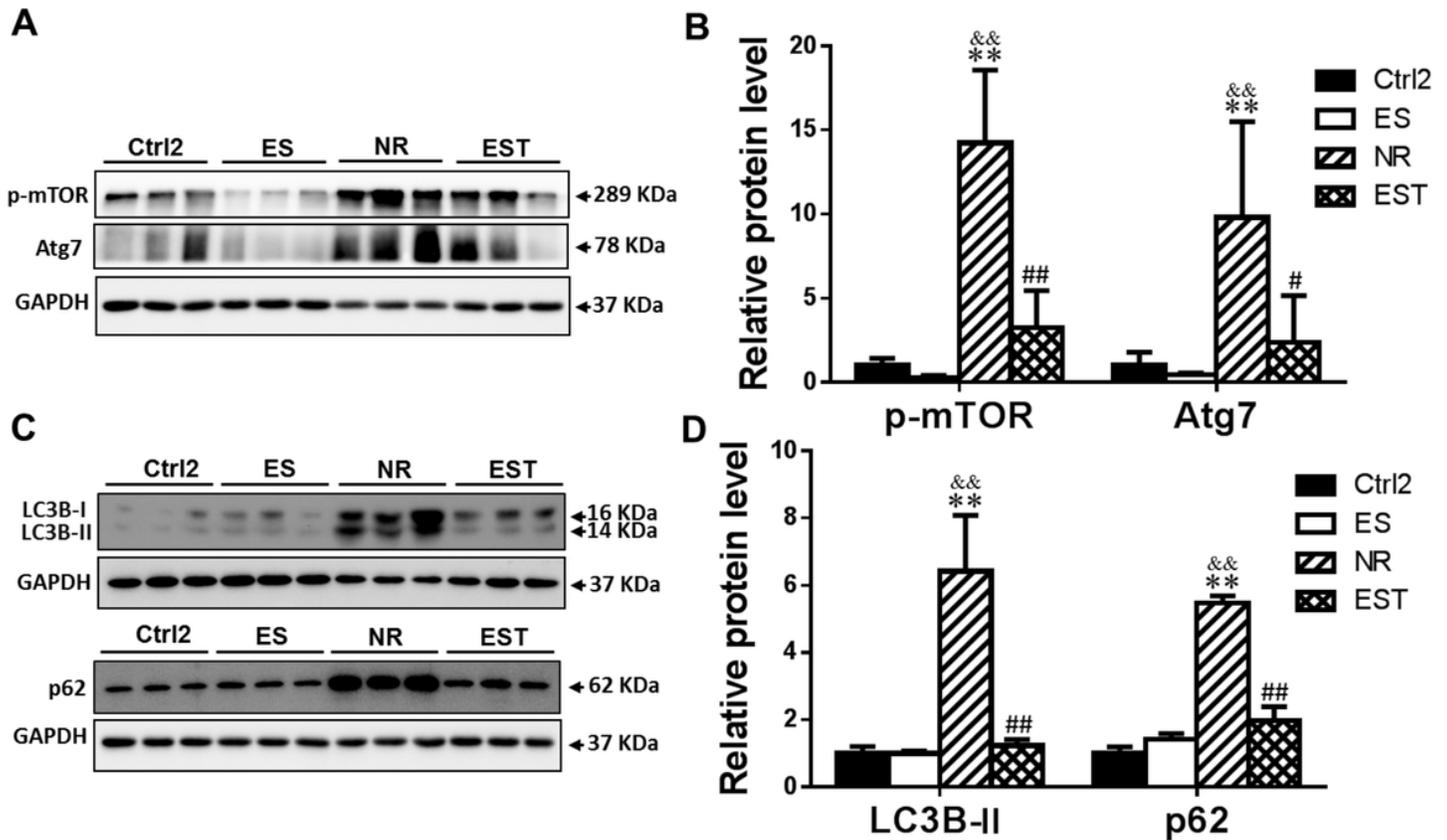
**Figure 3**

Immobilization causes the increment of LC3 puncta in rabbit skeletal muscle. Male rabbits were immobilized with plaster bandage on left hind knee joint in extension position. Rectus femoris was dissected at 2, 4 and 6 weeks after immobilization. (A) Representative images from control 1 group and 4-weeks immobilization group using immunostaining for LC3B. Scale bars represent 20  $\mu$ m. (B) Quantitative analysis for immunostaining of LC3B. Data were expressed as mean  $\pm$  SD. n=6. Ctrl1: control 1 group; I-4: immobilization for 4 weeks. \*P < 0.05 compared to the Ctrl1.



**Figure 4**

Low-frequency electrical stimulation improves disuse muscle atrophy and knee joint contracture. Male rabbits were treated with low-frequency electrical stimulation on the quadriceps femoris muscle for 3 weeks after 4 weeks of immobilization. Rectus femoris was dissected after 3 weeks of electrical stimulation. (A) Knee joint range of motion (ROM). (B) Representative images from the cross section of skeletal muscle fibers using H&E staining. Scale bars represent 50 μm. (C) Quantitative analysis for cross-sectional area (CSA) of skeletal muscle fibers. Ctrl2: control 2 group; ES: electrical stimulation group; NR: natural recovery group; EST: electrical stimulation treatment group. Data were expressed as mean ± SD. n=6. \*\*P<0.01 compared to the Ctrl2; &&P<0.01 compared to the ES; #P<0.05, ##P<0.01 compared to the NR.



**Figure 5**

Low-frequency electrical stimulation alleviates immobilization-triggered activation of autophagy in rabbit skeletal muscle. Male rabbits were treated with low-frequency electrical stimulation on the quadriceps femoris muscle for 3 weeks after 4 weeks of immobilization. Rectus femoris was dissected after 3 weeks of electrical stimulation. (A) p-mTOR and Atg7 proteins were detected using western blotting. (B) Quantitative analysis for p-mTOR and Atg7. (C) LC3B-I/II and p62 proteins were detected using western blotting. (D) Quantitative analysis for LC3B-II and p62. Data were expressed as mean ± SD. n=6. Ctrl2: control 2 group; ES: electric stimulation group; NR: natural recovery group; EST: electrical stimulation treatment group. Data were expressed as mean ± SD. n=6. \*\*P < 0.01 compared to the Ctrl2; &&P < 0.01 compared to the ES; #P < 0.05, ##P < 0.01 compared to the NR.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementarytables.docx](#)