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Original Article

Attenuation of skeletal muscle atrophy via acupuncture, electro-acupuncture, and electrical stimulation

T. Brock Symons [a,1], Jinho Park [a,1], Joo Hyun Kim [a], Eun Hye Kwon [a], Jesse Delacruz [a], Junghoon Lee [b], Yoonjung Park [b], Eunhee Chung [c], Sukho Lee [a,*]

[a] Department of Counselling, Health, and Kinesiology, Texas A&M University-San Antonio, San Antonio, TX, U.S.A
[b] Department of Health and Human Performance, University of Houston, Houston, TX, U.S.A
[c] Department of Kinesiology, The University of Texas at San Antonio, San Antonio, TX, U.S.A

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ABSTRACT

Background: Accelerated skeletal muscle wasting is a shared trait among many pathologies and aging. Acupuncture has been used as a therapeutic intervention to control pain; however, little is known about its effects on skeletal muscle atrophy and function. The study's purpose was to compare the effects of acupuncture, electro-acupuncture, and electrical stimulation on cast-induced skeletal muscle atrophy.
Methods: Forty female Sprague Dawley rats were randomly divided into groups: Control, casted (CAST), CAST+Acupuncture (CAST-A), 4) CAST+Electro-acupuncture (CAST-EA), and CAST+Electrical stimulation (CAST-ES) ($n = 8$). Plaster casting material was wrapped around the left hind limb. Acupuncture and electro-acupuncture (10 Hz, 6.4 mA) treatments were applied by needling acupoints (stomach-36 and gallbladder-34). Electrical stimulation (10 Hz, 6.4 mA) was conducted by needling the lateral and medial gastrocnemius muscles. Treatments were conducted for 15 min, three times/week for 14 days. Muscle atrophy F-box (MAFbx), muscle RING finger 1 (MuRF1), and contractile properties were assessed.
Results: Fourteen days of cast-immobilization decreased muscle fiber CSA by 56% in the CAST group ($p = 0.00$); whereas, all treatment groups demonstrated greater muscle fiber CSA than the CAST group ($p = 0.00$). Cast-immobilization increased MAFbx and MuRF1 protein expression in the CAST group ($p<0.01$) while the CAST-A, CAST-EA, and CAST-ES groups demonstrated lower levels of MAFbx and MuRF1 protein expression ($p<0.02$) compared to the CAST group. Following fourteen days of cast-immobilization, peak twitch tension did not differ between the CAST-A and CON groups ($p = 0.12$).
Conclusion: Skeletal muscle atrophy, induced by 14 days of cast-immobilization, was significantly attenuated by acupuncture, electro-acupuncture, or electrical stimulation.

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1. Introduction

Accelerated skeletal muscle wasting is a shared trait among many pathologies (cachexia) including congestive heart failure, cancer, diabetes, liver cirrhosis, chronic infection, and renal and pulmonary insufficiency.[1] Furthermore, the progressive loss of skeletal muscle mass and strength is a central characteristic of healthy aging (sarcopenia). Reduced skeletal muscle mass and function brought on by disease and/or aging diminishes one's ability to counter illness or injury, exacerbates the prognosis of many diseases, and greatly increases morbidity and mortality.[2] Despite the high prevalence of individuals suffering from skeletal muscle impairment, there is no effective treatment for skeletal muscle wasting and compromised function; and further, skeletal muscle remains an under-treated organ.[3]

The loss of skeletal muscle mass is a critical issue for patients who cannot perform physical activity, such as individuals confined to bed rest and/or cast-immobilization, as the absence of skeletal muscle loading leads to further skeletal muscle atrophy. Cast-immobilization is a conventional treatment for orthopedic disorders such as ligament injuries, bone fractures or dislocations and results in significant disuse skeletal muscle atrophy.[4] Disuse skeletal muscle atrophy causes numerous detrimental morphological and functional changes to skeletal muscle tis-

* Corresponding author at: Department of Counselling, Health, and Kinesiology, Texas A&M University-San Antonio, One University Way, STEC 142A, San Antonio, TX 78224, U.S.A.
E-mail address: slee@tamusa.edu (S. Lee).
[1] These two authors contributed equally to this work.

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sue. These changes are created by the resulting decrease in protein synthesis and the accompanying increase in protein degradation which leads to a significant reduction in skeletal muscle mass.[5] At the muscle fiber level, disuse muscle atrophy causes a significant decrease in fiber cross-sectional area via sarcomere termination, endothelial disruption, connective tissue accumulation amid fibers, apoptotic myonuclei, and diminished capillary density.[6, 7] Functional changes related to skeletal muscle disuse atrophy caused by cast-immobilization include reductions in range of motion, rate of contractile force development, and maximal isometric strength, impaired neuromuscular activation, and the formation myogenic contracture via decreased muscle length and increased passive stiffness.[8-10] Mechanical loading is essential for the conservation of skeletal muscle mass; however, physical activity such as resistance exercise is not always feasible under certain physio-pathophysiological conditions.[11] Additionally, there is currently no reliable pharmacological intervention to prevent skeletal muscle atrophy.[12] Therefore, it is imperative to develop alternative treatments that prevent and/or inhibit the loss of skeletal muscle following skeletal muscle disuse.

Acupuncture is a common and frequently used therapy in complementary and alternative medicine.[13] Acupuncture exerts its therapeutic effects through the insertion of a needle at a specific acupuncture point and thus, altering the qi (energy) and blood circulation along the meridian.[14] Although acupuncture has been used predominantly to manage pain under pathological conditions,[15, 16] recent studies suggest that acupuncture can be used for the treatment of musculoskeletal disorders.[17] Nagaoka et al.[18] and Shinbara et al.[17] both demonstrated that blood flow within the skeletal muscle can be significantly increased following acupuncture. Acupuncture has further been shown to reduce exercise-induced muscle soreness[19] and attenuate inflammation at various joints throughout the body such as the elbow,[16] the knee,[20] and the shoulder joint.[21] Acupuncture has also been shown to have an influence on skeletal muscle function demonstrating the effectiveness of a single bout of acupuncture in increasing quadriceps muscle strength.[22] Furthermore, Gao et al.[23] demonstrated that acupuncture had a protective effect on skeletal muscle from injury and enhanced skeletal muscle performance.

More specific to the current study is the finding that acupuncture at gallbladder 34 had a protective effect on skeletal muscle atrophy following immobilization.[24] Further, Onda et al.[25] demonstrated that acupuncture of the gastrocnemius muscle partially prevented skeletal muscle atrophy in mice. Lastly, the Wang group provides support for acupuncture, in addition to low-frequency electrical stimulation, and the neutralization of skeletal muscle atrophy.[26, 27] To date, no study to our knowledge has determined which treatment, acupuncture, electro-acupuncture or skeletal muscle electrical stimulation, is the most effective at attenuating and/or preventing the loss of skeletal muscle mass following skeletal muscle disuse.

We hypothesized that acupuncture, electro-acupuncture, and electrical stimulation would attenuate skeletal muscle atrophy induced by disuse. To test this hypothesis, we exposed Sprague-Dawley rats to acupuncture, electro-acupuncture, and electrical stimulation three times per week for two weeks during hind limb cast-immobilization and assessed skeletal muscle contractile properties and target proteins of atrophy.

2. Methods

2.1. Animals and experimental design

Female Sprague-Dawley rats (n = 40; nine weeks of age; weighting 205 ± 11 g; Charles River, Stone Ridge, NY, U.S.A) were assigned to one of five groups: 1) non-casted (CON, n = 8); 2) casted (CAST, n = 8); 3) casted receiving acupuncture (CAST-A, n = 8); 4) casted receiving electro-acupuncture (CAST-EA, n = 8); and 5) casted receiving electrical stimulation (CAST-ES n = 8). All experimental procedures were be approved by the Texas A&M University - San Antonio Institutional Animal Care and Use Committee (protocol reference no. 2020–06). The animals were housed under controlled temperature (22.8 ± 0.3 °C) and lighting (12 – 12-hour light-dark cycle) with food and water ad libitum.

After a five-day acclimation period, the animals were randomly divided into five groups (CON, CAST, CAST-A, CAST-EA, and CAST-ES). The animals in the CAST, CAST-A, CAST-EA, and CAST-ES groups had their left hind limb immobilized via a plaster cast for 14 days. Twenty-four hours after cast immobilization, the animals in the CAST-A, CAST-EA, and CAST-ES groups received 15 min of acupuncture, electro-acupuncture, and electrical stimulation, respectively. The animals received six treatments (three times per week) during the 14-day immobilization period. Twenty-four hours after the removal of the plaster cast, the contractile properties of the plantaris muscle were assessed in all groups. Following the evaluation of contractile properties, the animals were euthanized, and the plantaris muscle was excised and immediately frozen in liquid nitrogen for biochemical analysis.

Animals were anesthetized with gaseous isoflurane (1–2 L /min) during the cast-immobilization procedure. A layer of pre-wrap tape (Johnson & Johnson, Skillman, NJ, U.S.A) was wrapped around the hind limb prior to casting to prevent skin abrasions. A plaster of paris cast (CraftWrap™, Goja, Miami, FL, U.S.A) was applied to the left hind limb two centimeters above the patella to the hind paw to ensure the ankle joint was fully plantar-flexed to induce atrophy of the plantaris muscle. To prevent the animals from chewing off and/or damaging the cast, bitter apple spray (Grannicks Bitter Apple, Norwalk, CT, U.S.A) was applied to the cast. Following the cast-immobilization procedure, the animals were monitored to confirm they were able to move freely within their cages and had free access to their food and water. Animals were inspected daily for abrasions, edema, and/or damage to the cast. The animal's hind limb was immobilized for 14 days.

2.2. Interventions

2.2.1. Acupuncture treatment

The acupuncture treatment involved needling (DBC™ Spring Ten, length 15 mm, diameter 0.16 mm, Lhasa OMS, Weymouth, MA, U.S.A) two acupuncture points of animal's left hind limb [stomach-36 (ST36) and gallbladder-34 (GB34)] to a depth of 0.3 to 0.5 mm. Acupuncture point ST36 was located by palpating below the knee and lateral to the lateral boarder of the tibial tuberosity of the hind limb. Gallbladder 34 was located by palpating the depression found anterior and inferior to the fibular head of the hind limb (Fig. 1). The acupuncture treatment was performed three times per week during the 14-day immobilization period for a duration of 15 min.

2.2.2. Electro-acupuncture treatment

The electro-acupuncture intervention employed the same needling strategy as the acupuncture treatment. Electrical current was delivered to the ST36 and GB34 acupuncture points using a portable ITO Physio-Therapy & Rehabilitation constant current stimulator (ES-160, ITO^{CO}, Nerima-Ku, Tokyo, Japan). The ES-160 unit delivered a constant waveform at a continuous frequency of 10 Hz, a phase duration of 150 μsec, and an intensity of 6.4 mA (Fig. 1). The electro-acupuncture treatment was performed three times per week during the 14-day immobilization period for a duration of 15 min.

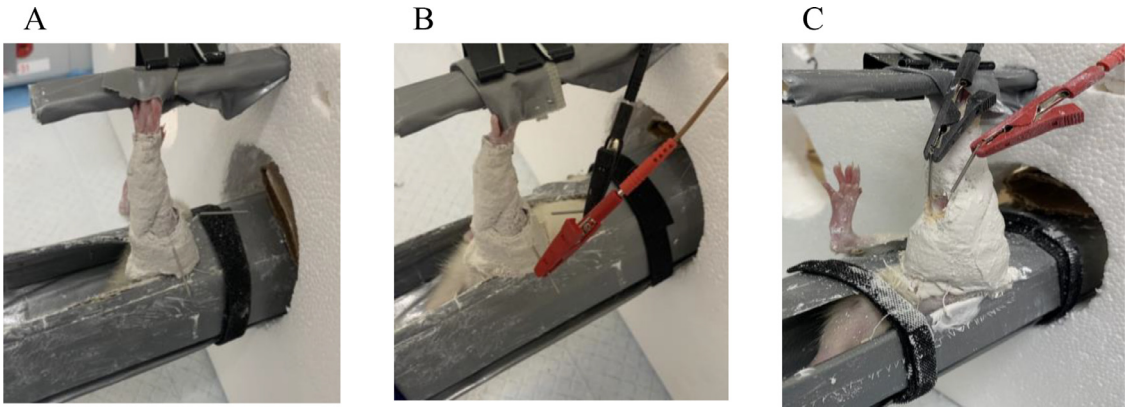

Fig. 1. Hind limb needle placement. A: acupuncture, B: electro-acupuncture, and C: electrical stimulation.

2.2.3. Electrical stimulation treatment

The electrical stimulation intervention employed the same electrical stimulation strategy as the electro-acupuncture treatment. However instead of providing the electrical current through acupuncture points ST36 and GB34, needle electrodes were directly inserted into the gastrocnemius skeletal muscle (Fig. 1). The electrical stimulation treatment was performed three times per week during the 14-day immobilization period for a duration of 15 min.

2.3. Muscle cross sectional area, contractile properties and biochemical measures

2.3.1. Muscle cross sectional area

Whole muscle cross sectional area of the plantaris muscle was calculated using the following formula: CSA (mm^2) = muscle mass (mg) * [1.06 (mg/mm^3) * muscle length (mm)].[28] Muscle fiber length (Lo) was estimated using the following formula: Lo (mm) = muscle mass length (mm) * 0.25.[28]

2.3.2. Contractile property assessment

Twenty-four hours after the 14-day cast-immobilization period, all animals were initially anesthetized with isoflurane (1–2 L/min) and then further anesthetized with a combination of ketamine (80 mg/kg) and xylazine (8 mg/kg) to induce a state of long-term anesthesia to assess skeletal muscle contractile properties. The contractile properties of the plantaris muscle were determined with a dual-mode servo and galvanometer (model 310C, Aurora Scientific, Richmond, ON, Canada). The dual mode servo output was interfaced with a computer (Dell Technologies, Round Rock, TX, U.S.A) equipped with a National Instruments A/D board (National Instruments Corp., Austin, TX, U.S.A). The data was stored and analyzed using Labview software (Version 3.0, National Instruments Corp., Austin, TX, USA).

The plantaris muscle was activated by stimulating the sciatic nerve with a silver wire electrode via an A-M System Isolated Pulse Stimulator (Model 2100, A-M System Inc., Sequim, WA, U.S.A). Peak twitch tension (Pt), contraction time and half-relaxation time (1/2Rt) were determined via a single twitch at 0.5 Hz, 7 V and a pulse duration of 0.5 ms.[29] Peak tetanic tension (Po) was ascertained from a single 330 ms train at 14 V. The intensity of the contraction was increased by increasing the frequency of stimulation from a starting frequency of 100 Hz until Po plateaued.[29]

Following the assessment of contractile properties, the animal was euthanized with an intracardiac injection consisting of a combination of ketamine (80 mg/kg) and xylazine (8 mg/kg). The plantaris muscle was removed and stored at −80 °C pending biochemical analysis.

2.3.3. Western blotting

Plantaris muscles were homogenized in a RIPA buffer with protease/phosphatase inhibitor cocktails and centrifuged at 14,000 rcf for 30 min at 4 °C. Protein concentration in the supernatants was quantified using the bicinchoninic acid assay. Muscle atrophy F-box (MAFbx) and muscle RING finger 1 (MuRF1) protein concentrations were assessed by Western blot analysis. Equal amounts of muscle protein (30 µg per sample) were passed through 8% SDS-PAGE (sodium dodecyl sulfate/polyacrylamide gel electrophoresis). Proteins were transferred to a nitrocellulose membrane (0.2 um), blocked in nonfat dry milk (5%) in Tris-buffered saline-Tween 20 (0.05% TBST) for 60 min at room temperature (RT), and incubated overnight at 4 °C with phosphospecific antibodies for MAFbx (SC-166,806, 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) and MuRF1 (SC-398,608, 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A). Excess primary antibody was removed by washing three times in 1 Tris-buffered saline 0.1% Tween 20, and membranes were incubated with m-IgGκ BP-HRP anti-mouse secondary antibody (SC-516,102, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A) at room temperature. Loading differences were controlled by reproving the blot with an antibody against GAPDH (D16H11, 1:5000, Rabbit IgG, Signaling Technology, Danvers, MA, U.S.A). Blots were developed with enhanced chemiluminescence (Thermo Fisher Scientific, Waltham, MA, U.S.A) for visualization, detected by ChemiDoc MP imager (Bio- Rad, Hercules, CA, U.S.A) and quantified using Image J. 1.53 s software (National Institutes of Health, Bethesda, MA, U.S.A).

2.3.4. Plantaris muscle fiber cross sectional area

For hematoxylin and eosin (H&E) staining, sections of the plantaris muscle were frozen in optimal cutting temperature compound (Scigen Tissue-Plus^{TM} O.C.T. Compound, Fisher Health Care, Houston, TX, U.S.A) following extraction. Samples were cut into 20 μm sections, mounted on slides, and dried for 24 h. Sections were stained using standard with H&E according to standard protocol and observed under X20 magnification (Leica Biosystem, Deer Park, IL, U.S.A). Muscle fiber cross-sectional area was determined by averaging the cross-sectional area of 20 fibers per animal using Image J program (National Institutes of Health, Bethesda, MD, U.S.A).

2.4. Statistical analysis

Data are presented as means ± standard deviations. Statistical analysis was performed using IBM SPSS Statistics (version 27; IBM Corp, Armonk, NY, U.S.A). Changes in outcome measures were analyzed using separate one-way analyses of variance. When a significant between groups effect was found, a Tukey's test was used for

Table 1
Body and plantaris muscle weights.

Characteristics	Group				
	CON ($n = 8$)	CAST ($n = 8$)	CAST-A ($n = 8$)	CAST-EA ($n = 8$)	CAST-ES ($n = 8$)
Pre Body Weight (g)	203.1 ± 7.2	205.3 ± 16.8	207.6 ± 11.9	201.1 ± 13.0	209.5 ± 9.0
Post Body Weight (g)	213.1 ± 6.2*	203.4 ± 15.1	202.3 ± 9.2	197.5 ± 11.1	208.8 ± 9.2

Values are means (± standard deviation).
* Significant difference between starting body weight and final body weight ($p < 0.05$). CON = control; CAST = cast; CAST-*A* = cast + acupuncture; CAST-EA = cast + electro-acupuncture; CAST-ES = cast + electrical stimulation.

Table 2
Plantaris muscle characteristics.

Characteristics	Group				
	CON ($n = 8$)	CAST ($n = 8$)	CAST-A ($n = 8$)	CAST-EA ($n = 8$)	CAST-ES ($n = 8$)
Muscle Weight (mg)	223.9 ± 20.9	144.0 ± 13.0*	156.7 ± 14.3*	161.5 ± 20.4*	160.2 ± 20.0*
Plantaris/body, x10^5	105.1 ± 9.8	71.0 ± 7.1*	77.4 ± 5.3*	81.8 ± 9.5*	76.7 ± 8.6*
Lo (mm)	9.1 ± 0.4	8.6 ± 0.3	8.8 ± 0.4	8.6 ± 0.3	8.8 ± 0.3
Plantaris CSA (mm^2)	1.06 ± 0.03	1.02 ± 0.06	1.04 ± 0.11	1.04 ± 0.05	1.05 ± 0.05
Fiber CSA (μm^2)	12,807.7 ± 1289.2	556.9 ± 1002.7*	8235.5 ± 1247.6*,†	9670.2 ± 1224.0*,†	8624.3 ± 734.8*,†

Values are means (± standard deviation).
CON = control; CAST = cast; CAST-A = cast + acupuncture; CAST-EA = cast + electro acupuncture; CAST-ES = cast + electrical stimulation.
* Significant difference from control group ($p < 0.05$).
† Significant difference from cast group ($p < 0.05$). Lo = Muscle Fiber Length; CSA = cross sectional area.

post-hoc analysis. Statistical significance for all analyses was set at $\alpha = 0.05$.

3. Results

3.1. Animal characteristics

Animal characteristics are presented in Table 1. No difference in body weight was found between groups prior to casting ($p = 0.26$) and following 14 days of cast-immobilization ($p = 0.05$). The CON group demonstrated a 4.9% increase in body weight across the 14-day immobilization period ($p = 0.01$); whereas, the treatment groups displayed a non-significant reduction in body weights ($p>0.05$).

3.2. Muscle characteristics

Plantaris muscle weights were significantly reduced in all groups following 14 days of cast- immobilization compared to the CON group ($p = 0.00$). The CAST-A, CAST-EA, and CAST-ES groups displayed attenuated losses in plantaris muscle weight but did not differ from the CAST group (Table 2). The ratio of plantaris muscle weight to body weight was determined as a measure of muscle wasting[27] and demonstrated a significant group effect ($p = 0.00$) across the 14-day cast-immobilization period. Compared to the CON group, all treatment groups displayed lower plantaris muscle weight to body weight ratios ($p = 0.00$). The CAST-A, CAST-EA, and CAST-ES groups demonstrated reduced muscle wasting but did not differ from the CAST group. There was no difference in muscle length and plantaris muscle CSA among the groups (Table 2). Despite no change in whole muscle CSA, muscle fiber CSA was different among the treatment groups ($p = 0.00$). The CAST group demonstrated a 56% drop in muscle fiber CSA compared to the CON group ($p = 0.00$). The CAST-A, CAST-EA, and CAST-ES groups displayed a 27.9%, 24.5%, and 32.7% reduction in muscle fiber CSA compared to the CON group, respectively ($p = 0.00$). Despite significant reductions in muscle fiber CSA when compared to the CON group, the CAST-A, CAST-EA, and CAST-ES groups demonstrated significantly greater muscle fiber CSA than the CAST group (CAST: 5561.9 ± 1002.7 vs. CAST-ACU: 9235.5 ± 1247.6, CAST-EACU: 9670.02 ± 1224.0, CAST-ESTM: 8624.33 ± 734.8; $p = 0.00$).

3.3. Biochemical measures of atrophy

MuRF1 demonstrated a significant group effect following 14 days of cast-immobilization ($p = 0.00$). Post hoc analysis revealed 14 days of casting significantly increased the expression of MuRF1 (CON: 0.69 ± 0.15 vs. CAST: 1.26 ± 0.44**;** $p = 0.00$) in the plantaris muscle (Fig. 2). Compared to the CAST group, the CAST-A, CAST-EA, and CAST-ES groups demonstrated significantly lower levels of MuRF1 protein expression (CON: 0.69 ± 0.15 vs. CAST-A: 0.82 ± 0.20, CAST-EA: 0.73 ± 0.14, CAST-ES: 0.85 ± 0.21; $p<0.02$).

Fourteen days of cast-immobilization also induced a significant group effect for MAFbx protein expression ($p = 0.00$). MAFbx protein expression demonstrated a significant increase in CAST group when compared to CON group (1.24±0.31 vs. 0.57±0.20; $p = 0.00$) (Fig. 2). Further, the CAST-A, CAST-EA, and CAST-ES groups displayed significantly lower MAFbx protein expression when compared to the CAST group (CAST: 1.24±0.31 vs. CAST-A: 0.65 ± 0.23, CAST-EA: 0.52 ± 0.23 and CAST-ES: 0.76 ± 0.43**;** $p<0.02$).

3.4. Contractile properties of the plantaris muscle

Table 3 shows the contractile properties of the plantaris muscle. There was no difference in contraction time ($p = 0.30$) and half-relaxation time ($p = 0.84$) following the 14-day cast-immobilization period among the groups. Fourteen days of cast-immobilization decreased peak twitch tension in the CAST (−49.3%), CAST-EA (−38.8%), and CAST-ES (−26.4%) groups compared to the CON group. Peak twitch tension did not differ between the CAST-A and CON groups (CON: 2.01 ± 0.45 N vs. CAST-A: 1.60 ± 0.45 N; $p = 0.123$) following 14 days of cast-immobilization. In addition, the CAST-A group generated greater peak twitch tension (56.9%, $p = 0.013$) and specific peak twitch tension (46.2%, $p = 0.026$) than the CAST group following cast-immobilization (Table 3). Peak tetanic tension was significantly reduced ($p<0.025$) following cast-immobilization in the CAST, CAST-A, and CAST-EA groups compared to the CON group; whereas, the CAST-ES group did not differ from the CON group. No difference

Table 3
Contractile properties of the plantaris muscle.

	Group				
Characteristics	CON (n = 8)	CAST (n = 8)	CAST-A (n = 8)	CAST-EA (n = 8)	CAST-ES (n = 8)
CT (ms)	107.6 ± 45.5	132.9 ± 43.0	127.6 ± 67.8	135.5 ± 44.1	167.6 ± 24.0
1/2RT (ms)	216.6 ± 90.3	232.9 ± 86.1	242.5 ± 93.6	260.1 ± 40.7	245.3 ± 39.7
Pt (N)	2.01 ± 0.45	1.02 ± 0.30*	1.60 ± 0.45†	1.23 ± 0.21*	1.48 ± 0.16*
SPt (N/cm^2)	8.8 ± 2.5	6.5 ± 1.5	9.5 ± 2.6†	7.0 ± 0.8	8.7 ± 1.7
Po (N)	5.56 ± 2.57	3.20 ± 0.84*	3.34 ± 0.57*	3.31 ± 0.97*	4.13 ± 1.19
SPo (N/ cm^2)	8.7 ± 2.3	10.1 ± 1.9	9.5 ± 2.4	10.1 ± 2.4	11.1 ± 2.5

Values are means (± standard deviation).
* Significant difference from control group (p< 0.05).
† Significant difference from cast group (p< 0.05). CT = contraction time; 1/2RT = half relaxation time; Pt = peak twitch tension, Po = peak tetanic tension; SPt = specific peak twitch tension; SPo = specific peak tetanic tension; CON = control; CON = control; CAST = cast; CAST-*A* = cast + acupuncture; CAST-EA = cast + electro-acupuncture; CAST-ES = cast + electrical stimulation.

was found among the groups for specific tetanic tension following cast-immobilization.

4. Discussion

The present study hypothesized that acupuncture, electro-acupuncture, and electrical stimulation performed during 14 days of cast-immobilization would attenuate skeletal muscle atrophy and minimize the reduction in skeletal muscle contractile properties. The results of the study indicate that skeletal muscle atrophy was significantly reduced when acupuncture, electro-acupuncture, and electrical stimulation were performed during the 14-day casting period. We found that MAFbx and MuRF1 protein expressions were significantly reduced (range 33% to 58%) by acupuncture, electro-acupuncture, and electrical stimulation when compared to the CAST group. Additionally, CAST-A, CAST-EA, and CAST-ES significantly attenuated the loss of muscle fiber CSA in the plantaris skeletal muscle. Furthermore, our findings also revealed that acupuncture significantly attenuated the loss of peak twitch tension and electrical stimulation reduced the loss of peak tetanic tension in comparison to the CAST group following 14 days of cast-immobilization.

Cast-immobilization and the resulting mechanical unloading permit important clinical insights to be elucidated, as cast-immobilization leads to a rapid and consistent loss of skeletal muscle mass in rodents.[30] Two ubiquitin ligases found in skeletal muscle, MAFbx and MuRF1, are upregulated in different models of skeletal muscle atrophy and are responsible for the increased protein breakdown via the ubiquitin-proteasome system.[31-33] Our results support the elevation of MAFbx and MuRF1 protein expression following 14 days of cast-immobilization demonstrating a 117% and 84% increase, respectively. These findings are supported by Cruz et al.[30] who found that MAFbx protein levels were increased by seven days of cast-immobilization and by Lee et al.[34] who found that MAFbx and MuRF1 were elevated following five-weeks of cast-immobilization. Thereby conferring that the reduced mechanical loading induced by the 14-day cast-immobilization period increased the rate of skeletal muscle proteolysis in our animals.

Therapeutic interventions aimed at reducing skeletal muscle atrophy following disuse have demonstrated limited success in humans[27] with exercise representing the most valid treatment option.[12, 35] However, exercise is often not a realistic option for those who are bedridden or suffering from disease and thus, non-exercise-based interventions are required.[27] Importantly, our results revealed that acupuncture, electro-acupuncture, and electrical stimulation interventions successfully maintained MAFbx and MuRF1 protein expression levels during 14 days of cast-immobilization. Our findings are supported by Onda et al.[25] who found that two weeks of acupuncture and electro-acupuncture during hind limb suspension significantly decreased MAFbx mRNA expression in the soleus muscle of mice when compared to the hind limb suspension group. Unlike our results, the acupuncture and electro-acupuncture treatments employed by Onda et al.[25] during hind limb suspension period were not able to preserve MAFbx mRNA expression to the same level as their control group. Additionally, Onda et al.[25] showed that two weeks of acupuncture and electro-acupuncture significantly attenuated MuRF1 mRNA expression induced by hind limb suspension. The above findings demonstrate that acupuncture, electro-acupuncture, and electrical stimulation may be an appealing non-exercise-based intervention via the downregulation MAFbx and MuRF1 protein and mRNA expression to combat skeletal muscle disuse wasting.

Skeletal muscle wasting encompasses the loss of skeletal muscle protein and the regeneration capacity of muscle fibers. Acupuncture and electro-acupuncture represent exciting interventions that have the potential to combat skeletal muscle wasting on both fronts. First, by ameliorating the loss of skeletal muscle protein by decreased expression of atrophic genes and proteins; and second, by stimulating the regeneration of skeletal muscle fibers.[25,27] It has been demonstrated that daily acupuncture of the GB34 acupoint across 14 days of cast-immobilization had a positive effect on immobilization-induced skeletal muscle apoptosis.[24] Kim[24] found that acupuncture of the gastrocnemius muscle decreased the immunoreactivity of BAX and increased immunoreactivity of Bcl-2 when compared to a control group. Furthermore, Ameis et al.[36] investigated the therapeutic potential of a single session of acupuncture (ST36) and confirmed the presence of activated satellite cells within the tibialis anterior muscle. Lastly, Su et al.[26,27] investigated the effectiveness of acupuncture in combination with low frequency electric stimulation (electro-acupuncture) on diabetic- and denervation-induced skeletal muscle atrophy. Diabetes- and denervation-induced skeletal muscle atrophy were reduced by electro-acupuncture via improved muscle regeneration capacity due to IGF-1 upregulation, downregulation of myostatin, and increased expression of microRNAs.[26,27] Taken together, these results illustrate the potential of both acupuncture and electro-acupuncture as non-pharmaceutical and non-exercise-based intervention in the treatment of skeletal muscle wasting through the prevention of skeletal muscle protein degradation and the enhancement of skeletal muscle regeneration.

Changes in skeletal muscle weight and the ratio of plantaris muscle weight to body weight were quantified to assess skeletal muscle wasting. Our results revealed that 14 days of cast-immobilization decreased the above parameters in all treatment groups compared to the control group and are in agreement with other studies.[25,27,30,37] Despite finding significantly reductions in biochemical markers of skeletal muscle atrophy (MuRF1

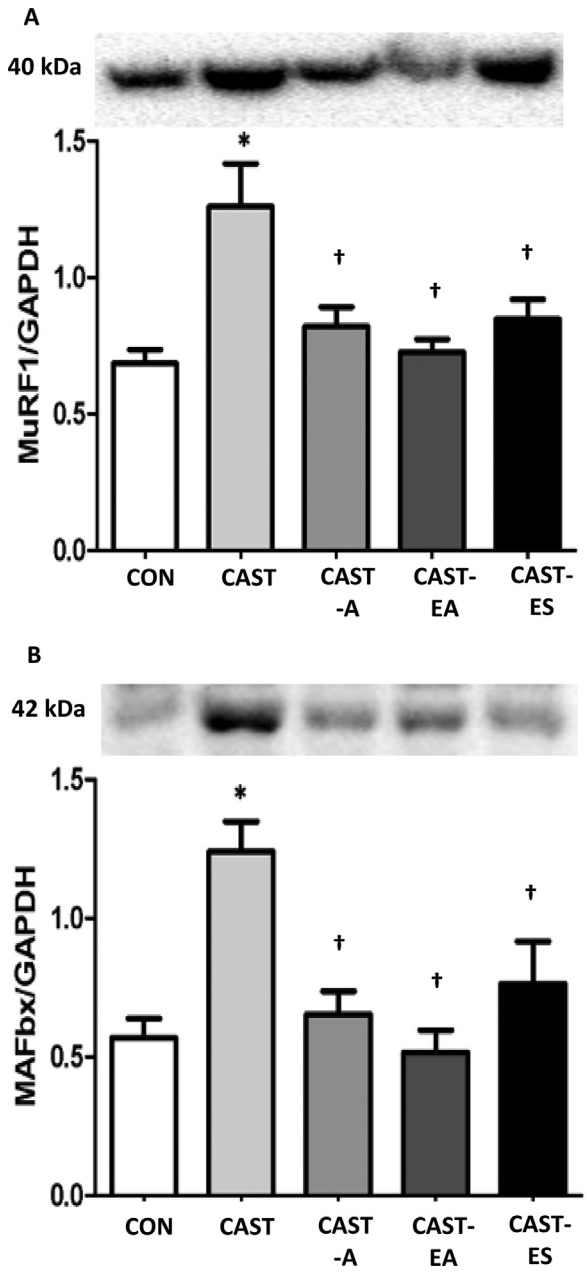

Fig. 2. Mean (±standard deviation) change in protein expression. A: muscle RING finger 1 (MuRF1) and B: muscle atrophy F box (MAFbx). *Significant difference from control group ($p < 0.05$). †Significant difference from cast group ($p < 0.05$). CON = control; CAST = cast; CAST-*A* = cast + acupuncture; CAST-EA = cast + electro-acupuncture; CAST-ES = cast + electrical stimulation.

and MAFbx), acupuncture, electro-acupuncture, and electrical stimulation failed to prevent or attenuate our measures of muscle wasting. This is in contrast to Su et al.[27] who found that 15 min of electro-acupuncture in mice across 14 days of denervation-induced atrophy significantly reduced the loss of plantaris muscle weight and the plantaris muscle weight to body weight ratio. Furthermore, Onda et al.[25] demonstrated that both daily acupuncture and electro-acupuncture significantly improved soleus muscle weight to body weight ratio following two weeks of hind limb suspension, albeit the amount of improvement in the soleus muscle weight to body weight ratio was insufficient when compared to the control group (as observed in the current study). Methodological differences between the Su et al.,[27] Onda et al.,[25] and the current study could account for the discrepancies in results. Despite using the same acupuncture points (GB34 and ST36) as Su et al.,[27] their electro-acupuncture treatment utilized a higher stimulation frequency (20 Hz vs. 10 Hz) and employed a more aggressive dosing strategy than the current study (15 min. daily for 14 days vs. 15 min., 3 times/week for 14 days). Similarly, Onda et al.[25] provided 30 min of daily acupuncture and electro-acupuncture over 14 days of hind limb suspension. Additionally, Onda et al.[25] directly needled the gastrocnemius muscle whereas we applied our needles to specific acupuncture points (ST36 and GB34). Despite varying outcomes among studies, both acupuncture and electro-acupuncture present a potential supplementary treatment for attenuating skeletal muscle wasting.

Wasting at the whole muscle level is the sum of changes in the cross-sectional area of individual skeletal muscle fibers. We found, as have others,[4,37,38] that cast-induced immobilization resulted in significant skeletal muscle fiber atrophy (57% reduction). Notably, our results revealed that six treatments of acupuncture, electro-acupuncture, and electrical stimulation across 14 days of cast-immobilization significantly attenuated the loss of plantaris muscle fiber CSA when compared to the casted group. Onda et al.[25] reported CSA of the soleus muscle fibers was improved by electro-acupuncture in hind limb suspended mice; however, they did not find significant improvements in gastrocnemius muscle fiber CSA with electro-acupuncture. This is in contrast to our result where electro-acupuncture significantly attenuated plantaris muscle fiber CSA following cast-immobilization. Fiber type composition between the soleus (predominantly type 1) and the gastrocnemius/plantaris (predominately type II) may have influenced the results; however this is not likely, as cast-immobilization can lead to equivalent levels of atrophy in both slow- and fast-twitch muscles.[39] Of further interest, Onda et al.[25] provided a more aggressive treatment option with daily 30-minute sessions of electro-acupuncture across 14 days of hind limb suspension verse our six, 15-minute sessions over 14 days. Despite a less aggressive treatment strategy, we also demonstrated that acupuncture (and electrical stimulation) significantly attenuated the loss of plantaris muscle fiber CSA following disuse which differs with Onda et al.[25] who found that manual acupuncture did not attenuate reductions in soleus muscle CSA induced by hind limb suspension. Importantly, our result show that acupuncture or electro-acupuncture can be employed as a potential treatment to diminish the effects of cast-immobilization on skeletal muscle fiber CSA.

Cast-immobilization and the resulting reduction in skeletal muscle fiber CSA can negatively affect skeletal muscle function and strength.[40] The current study revealed that 14 days of cast-immobilization reduced peak twitch force by 49% which is consistent with Belova et al.[41] who found a 38% decrease in twitch force following 7 days of unloading. A significant treatment effect was observed in the CAST-A group for peak twitch force. No difference in peak twitch force was found between the CON and the CAST-A group following 14 days of cast-immobilization, demonstrating a noteworthy prophylactic effect on plantaris muscle function. This was not the case for the CAST-EA and CAST-ES groups who demonstrated significant reductions in peak twitch force when compared to the CON group and also, did not differ from the CAST group after 14 days of cast-immobilization. However, this optimistic finding for the CAST-A group must be interpreted with caution as the CAST-A group demonstrated a significant reduction in peak tetanic force following cast-immobilization. The reason for the discrepancy is not clear.

Fourteen days of cast-immobilization significantly reduced peak tetanic force by 42%, 39%, and 41% in the CAST, CAST-A, and CAST-EA groups; respectively. In contrast the CAST-ES group did not differ from the CON group, as peak tetanic force was maintained across the 14-day cast-immobilization period. The reduction in peak tetanic force exhibited by the CAST group was anticipated

and paralleled previous work.[41] The discrepancy in findings between the CAST-ES group and the CAST-A and the CAST-EA (despite receiving the same electrical stimulation protocol) groups is intriguing. The discrepancy may be explained by the location of the stimulation, which differed between the two groups. The CAST-EA group was stimulated through the ST36 and GB34 acupuncture points (located just below the knee) which may have reduced the magnitude of the stimulus and perhaps the percentage of plantaris muscle fibers activated throughout the treatment. In contrast, the CAST-ES group was stimulated via the belly of the medial and lateral gastrocnemius muscle which may have resulted in the plantaris muscle receiving a larger stimulus and potentially activating a greater number of plantaris muscle fibers during the treatment. The magnitude and the excitation of muscle fibers is principally dependent on the proximity of the stimulating electrode;[42] therefore, the electrical stimulation performed in the current study may have been a more effective treatment.

In summary, this study revealed that skeletal muscle atrophy, induced by 14 days of cast-immobilization, was significantly attenuated by acupuncture, electro-acupuncture or electrical stimulation. Furthermore, we have demonstrated that acupuncture significantly attenuated the loss of peak twitch tension and electrical stimulation reduced the loss of peak tetanic tension following 14 days of cast-immobilization. Our results suggest that the implementation of acupuncture, electro-acupuncture, or electrical stimulation may be used as potential therapeutic interventions for attenuating the loss of skeletal muscle mass and function that is associated with disuse skeletal muscle atrophy under different pathological conditions and aging.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Ethical statement

This research was approved by the institutional animal care and use committee (IACUC number: 2020–06).

Data availability

The data will be made available by the corresponding author upon reasonable request.

CRediT authorship contribution statement

T. Brock Symons: Investigation, Writing – original draft, Writing – review & editing. **Jinho Park:** Investigation, Writing – original draft, Writing – review & editing. **Eun Hye Kwon:** Investigation, Data curation, Writing – review & editing. **Jesse Delacruz:** Investigation, Data curation. **Junghoon Lee:** Writing – original draft. **Yoonjung Park:** Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Supervision. **Eunhee Chung:** Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Supervision. **Sukho Lee:** Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing, Supervision.

References

1. Holeček M. Beta-hydroxy-beta-methylbutyrate supplementation and skeletal muscle in healthy and muscle-wasting conditions. *J Cachexia Sarcopenia Muscle*. 2017;8(4):529–541.
2. Vainshtein A, Sandri M. Signaling pathways that control muscle mass. *Int J Mol Sci*. 2020;21(13):4759.
3. Furrer R, Handschin C. Muscle wasting diseases: novel targets and treatments. *Annu Rev Pharmacol Toxicol*. 2019;59:315.
4. Nozaki R, Hung Y-L, Takagi K, Nakano D, Fujii T, Kawanishi N, et al. Differential protective effects of Radix astragali, herbal medicine, on immobilization-induced atrophy of slow-twitch and fast-twitch muscles. *Biomed Res*. 2020;41(3):139–148.
5. Mostafa A, Samir S. Role of melatonin on immobilization induced effects in rat soleus muscle. *Bull Egyptian Soc Physiol Sci*. 2014;34(1):104–120.
6. Pierno S, Liantonio A, Camerino GM, De Bellis M, Cannone M, Gramegna G, et al. Potential benefits of taurine in the prevention of skeletal muscle impairment induced by disuse in the hindlimb-unloaded rat. *Amino Acids*. 2012;43(1):431–445.
7. Bataa M, Kalleny NK, Hamam GG. Effect of amino acid L-leucine on the musculo-skeletal changes during cast-immobilization in adult male albino rats. Physiological and Histological study. *Life Sci J*. 2011;8(2).
8. Hvid L, Aagaard P, Justesen L, Bayer ML, Andersen JL, Ørtenblad N, et al. Effects of aging on muscle mechanical function and muscle fiber morphology during short-term immobilization and subsequent retraining. *J Appl Physiol*. 2010;109(6):1628–1634.
9. Ohira T, Kawano F, Ohira T, Goto K, Ohira Y. Responses of skeletal muscles to gravitational unloading and/or reloading. *The J Physiol Sci*. 2015;65(4):293–310.
10. Kaneguchi A, Ozawa J, Minamimoto K, Yamaoka K. Morphological and biomechanical adaptations of skeletal muscle in the recovery phase after immobilization in a rat. *Clin Biomech*. 2020;75:104992.
11. Fujitani M, Mizushige T, Kawabata F, Uozumi K, Yasui M, Hayamizu K, et al. Dietary Alaska pollack protein improves skeletal muscle weight recovery after immobilization-induced atrophy in rats. *PLoS ONE*. 2019;14(6):e0217917.
12. Adams GR, Haddad F, Bodell PW, Tran PD, Baldwin KM. Combined isometric, concentric, and eccentric resistance exercise prevents unloading-induced muscle atrophy in rats. *J Appl Physiol*. 2007;103(5):1644–1654.
13. Astin JA, Marie A, Pelletier KR, Hansen E, Haskell WL. A review of the incorporation of complementary and alternative medicine by mainstream physicians. *Arch Intern Med*. 1998;158(21):2303–2310.
14. Chae Y, Olausson H. The role of touch in acupuncture treatment. *Acupunct Med*. 2017;35(2):148–152.
15. Lin R-T, Tzeng C-Y, Lee Y-C, Ho W-J, Cheng J-T, Lin J-G, et al. Acute effect of electroacupuncture at the Zusanli acupoints on decreasing insulin resistance as shown by lowering plasma free fatty acid levels in steroid-background male rats. *BMC Complement Altern Med*. 2009;9(1):1–9.
16. Trinh K, Graham N, Gross A, Goldsmith C, Wang E, Cameron I, et al. Acupuncture for neck disorders. *Spine*. 2007;32(2):236–243.
17. Shinbara H, Nagaoka S, Izutani Y, Okubo M, Kimura K, Mizunuma K, et al. Contribution of adenosine to the increase in skeletal muscle blood flow caused by manual acupuncture in rats. *Acupunct Med*. 2017;35(4):284–288.
18. Nagaoka S, Shinbara H, Okubo M, Kawakita T, Hino K, Sumiya E. Contributions of ADP and ATP to the increase in skeletal muscle blood flow after manual acupuncture stimulation in rats. *Acupunctu Med*. 2016;34(3):229–234.
19. Hübscher M, Vogt L, Bernhörster M, Rosenhagen A, Banzer W. Effects of acupuncture on symptoms and muscle function in delayed-onset muscle soreness. *The J AlternComplement Med*. 2008;14(8):1011–1016.
20. Witt C, Brinkhaus B, Jena S, Linde K, Streng A, Wagenpfeil S, et al. Acupuncture in patients with osteoarthritis of the knee: a randomised trial. *The Lancet*. 2005;366(9480):136–143.
21. Kleinhenz J, Streitberger K, Windeler J, Güßbacher A, Mavridis G, Martin E. Randomised clinical trial comparing the effects of acupuncture and a newly designed placebo needle in rotator cuff tendinitis. *Pain*. 1999;83(2):235–241.
22. Hübscher M, Vogt L, Ziebart T, Banzer W. Immediate effects of acupuncture on strength performance: a randomized, controlled crossover trial. *Eur J Appl Physiol*. 2010;110(2):353–358.
23. Gao M, Yang H-Y, Liu T-Y, Kuai L. Effects of manual acupuncture and electroacupuncture on mitochondria of skeletal muscle cells in rats of acute swimming exercise. *Zhongguo Zhen jiu= Chin Acupunct Moxibustion*. 2005;25(6):421–424.
24. Kim BH. The effects of acupuncture at GB34 on disuse muscle atrophy in rats. *J Korean Med Rehabil*. 2018;28(4):1–9.
25. Onda A, Jiao Q, Nagano Y, Akimoto T, Miyamoto T, Minamisawa S, et al. Acupuncture ameliorated skeletal muscle atrophy induced by hindlimb suspension in mice. *Biochem Biophys Res Commun*. 2011;410(3):434–439.
26. Su Z, Robinson A, Hu L, Klein JD, Hassounah F, Li M, et al. Acupuncture plus low-frequency electrical stimulation (Acu-LFES) attenuates diabetic myopathy by enhancing muscle regeneration. *PLoS ONE*. 2015;10(7):e0134511.
27. Su Z, Hu L, Cheng J, Klein JD, Hassounah F, Cai H, et al. Acupuncture plus low-frequency electrical stimulation (Acu-LFES) attenuates denervation-induced muscle atrophy. *J Appl Physiol*. 2016;120(4):426–436.
28. Lee S, Farrar RP. Resistance training induces muscle-specific changes in muscle mass and function in rat. *J Exercise physiolo online*. 2003;6(2).
29. Lee S, Hong K-S, Kim K. Effect of previous strength training episode and retraining on facilitation of skeletal muscle hypertrophy and contractile properties after long-term detraining in rats. *J Exerc Rehabil*. 2016;12(2):79.
30. Cruz A, Ferian A, Alves PK, Silva WJ, Bento MR, Gasch A, et al. Skeletal muscle anti-atrophic effects of leucine involve myostatin inhibition. *DNA Cell Biol*. 2020;39(12):2289–2299.
31. Okamoto T, Torii S, Machida S. Differential gene expression of muscle-specific ubiquitin ligase MAFbx/Atrogin-1 and MuRF1 in response to immobilization-induced atrophy of slow-twitch and fast-twitch muscles. *The J Physiol Sci*. 2011;61(6):537–546.

32. Bodine SC, Latres E, Baumhueter S, Lai VK-M, Nunez L, Clarke BA, et al. Identification of ubiquitin ligases required for skeletal muscle atrophy. *ScienceScience*. 2001;294(5547):1704–1708.
33. Gomes MD, Lecker SH, Jagoe RT, Navon A, Goldberg AL. Atrogin-1, a muscle--specific F-box protein highly expressed during muscle atrophy. *Proceedings of the National Academy of Sciences*; 2001:14440–14445.
34. Lee J-B, Kwon D-K, Jeon Y-J, Mealworm Song Y-J. Tenebrio molitor)-derived protein supplementation attenuates skeletal muscle atrophy in hindlimb casting immobilized rats. *Chin J Physiol*. 2021;64(5):211.
35. Gielen S, Sandri M, Kozarez I, Kratzsch J, Teupser D, Thiery J, et al. Exercise training attenuates MuRF-1 expression in the skeletal muscle of patients with chronic heart failure independent of age: the randomized Leipzig Exercise Intervention in Chronic Heart Failure and Aging catabolism study. *CirculationCirculation*. 2012;125(22):2716–2727.
36. Ameis K, Kanaan YM, Das JR, George Jr M, Sobrian S. Effect of manual acupuncture-induced injury on rat skeletal muscle. *Med Acupunct*. 2008;20(4):225–230.
37. Suzuki H, Yoshikawa Y, Tsujimoto H, Kitaura T, Muraoka I. Clenbuterol accelerates recovery after immobilization-induced atrophy of rat hindlimb muscle. *Acta Histochem*. 2020;122(1):151453.
38. Usuki F, Fujimura M, Nakamura A, Nakano J, Okita M, Higuchi I. Local vibration stimuli induce mechanical stress-induced factors and facilitate recovery from immobilization-induced oxidative myofiber atrophy in rats. *Front Physiol*. 2019;10:759.
39. Machida S, Booth F. Changes in signalling molecule levels in 10-day hindlimb immobilized rat muscles. *Acta Physiol Scand*. 2005;183(2):171–179.
40. Tayel SG, Abdel-Hady El-Safty FE-N, El-Sherif N, Faried M. Histological structure of skeletal muscle in disuse muscle atrophy and role of exercise. *Egyptian J Histol*. 2021;44(1):1–7.
41. Belova SP, Kalashnikova EP, Tyganov SA, Kostrominova TY, Shenkman BS, Nemirovskaya TL. Effect of enhanced muscle tone on the expression of atrogenes and cytoskeletal proteins during postural muscle unloading. *Arch Biochem Biophys*. 2022:109291.
42. Guo Y, Phillips BE, Atherton PJ, Piasecki M. Molecular and neural adaptations to neuromuscular electrical stimulation; Implications for ageing muscle. *Mech Ageing Dev*. 2021;193:111402.