Exercise training improves the adipocyte accumulation and muscle fibrosis of the muscle by TGF-β1 and α-SMA reduction after botulinum toxin type A administration in mice

Esther Lee
Chung-Ang University
Song I Im
Chung-Ang University
Yu-jin Kim
Chung-Ang University
Su Young Kim
Chung-Ang University
Jungtae Na (pugokjebi@gmail.com)
Seoul Veterans Hospital https://orcid.org/0000-0003-1296-4010
Beom Joon Kim
Chung-Ang University

Research article

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Abstract

Background: We aimed to investigate the effect of treadmill exercise on functional recovery of the mouse gastrocnemius muscle and nerve after botulinum toxin type A (BoNT-A) administration.

Methods: After injecting 0.5 units of BoNT-A into the gastrocnemius muscle of ICR mice, treadmill exercise was carried out for a six-week period, after which the muscle volume, weight, and sciatic functional index (SFI) were obtained and nerve conduction study (NCS) and histological evaluation were performed.

Results: There was no change in the gastrocnemius weight and volume, but NCS and SFI increased after exercise. Exercise prevented induced adipocyte accumulation and muscle fibrosis. Moreover, TGF-β1 and α-SMA expression decreased and CD34, BDNF, and SNAP-25 expression increased when treadmill exercise was performed after BoNT-A injection.

Conclusions: The exercise was effective in the recovery of nerve function and would help in the recovery of muscle function by preventing accumulation of fat cells and muscle fibrosis after BoNT-A administration.

Background

Botulinum toxin type A (BoNT-A) is a neurotoxin that acts selectively on the neuromuscular junction[1]. BoNT-A cleaves the synaptosomal nerve-associated protein 25 (SNAP-25) protein, which helps in the exocytosis of vesicles containing neurotransmitters at the nerve endings[2]. Thus, the blocking of neurotransmitters by BoNT-A through the prevention of fusion of synaptic vesicles leads to muscle paralysis[3]. The effect of BoNT-A lasts for about 6 months after injection, and then muscle mass, muscle contraction, and nerve function are restored[4]. Currently, BoNT-A is widely used in dermatology for cosmetic purposes to reduce muscle size[5]. Exercise inhibits the degeneration of muscular function and improves neural function[6, 7]. In addition, exercise affects the growth of skeletal muscle by increasing muscle protein synthesis (MPS) and enhancing muscle function by preventing the replacement of muscle fibers with adipocyte and fibrosis[8, 9]. Numerous studies proved that treadmill exercise is effective on the functional side of muscles[10]. Treadmill is used in animal experiments to exert aerobic exercise effects[11]. In a study of peripheral nerve injury and recovery, treadmill exercise improved nerve and muscle response. There are limited studies on the changes in muscles and nerves by exercise after BoNT-A injection. In a rat exercise training model, 7 days of voluntary wheel running increased the mass and fiber size of BoNT-A-injected muscle without consistent changes in muscle production or number in juvenile rats[12]. Additionally, 3 weeks of running wheel exercise did not change the mass of BoNT-A- or saline-injected muscle and increased mechanosensing and signaling genes like titin, Ankrd2, and muscle LIM protein in BoNT-A-injected muscle[13].

However, the effect of interaction between exercise and BoNT-A administration has not been clearly demonstrated. In this study, we investigated the effect of treadmill exercise on muscular weight and the

functional recovery of muscles when sustained muscle contraction and relaxation were induced by treadmill exercise in muscles with blocked nerve transmission due to BoNT-A.

Methods

Animals

Seventy-two 6-week-old male ICR mice (25 ~ 30 g) were purchased from Orient Bio (Seongnam, Korea). The animals were fed standard solid feed (antibiotic-free) and water ad libitum and housed in sawdust-lined cages in an air-conditioned environment with a 12-hour light/dark cycle. The mice were anesthetized with Zoletil 50 (50 mg/kg) and xylazine (10 mg/kg). CO2 administration is used for euthanasia. 72 mice were separated into four groups: control, treadmill (saline), BoNT-A control (BC), and BoNT-A treadmill (BT) group. For control and saline groups, 10 μl of saline was injected into the right gastrocnemius muscle of each mouse, while 0.5 units of BoNT-A (Botox®; Allergan, Inc., Irvine, CA, USA) was diluted with 10 μl of saline and injected into the right gastrocnemius muscle of each mouse in the BC and BT groups. All animal procedures were approved by the Institutional Animal Care and Use Committee of Chung-Ang University (201700028) and confirmed to all applicable National Institutes of Health guidelines.

Treadmill Exercise

One week before starting the treadmill exercise, 72 mice were randomly assigned to four groups (18 mice per group) for 5 min of running at 50 m/min on a 45-cm treadmill belt to ensure that all mice performed similarly in treadmill work before BoNT-A administration. The treadmill used in these studies was a JD-A-09 treadmill manufactured by JEUNGDO Bio & Plant Co., Ltd. (Seoul, Korea). Mice of the two exercise groups (saline plus exercise and BoNT-A plus exercise) were run at the same time in the six-lane treadmill. Treadmill exercise was carried out for 20 min at a speed of 15 m/min at a temperature of 10 °C, five times a week over a 6-week period.

Nerve Conduction Study (ncs): Electrophysiology

A Dantec™ Keypoint® Focus (Natus Neurology, Middleton, WI, USA) instrument was used for nerve conduction recording of the gastrocnemius muscle (5 mice per group); the data were automatically analyzed and averaged. Three surface disc electrodes (recording anode, cathode, and ground electrode) were used. An incision was made from the gluteus muscles to the popliteal region to expose the sciatic nerve with standard settings (electric potential = 5.8 mA; stimulus duration = 0.1 ms). The proximal side of the nerve was stimulated with a supramaximal electric stimulation at the same position each time. The compound muscle action potential (CMAP) amplitude (mV) (peak to peak), distal latency (ms), and area (mm^2) were recorded each time. The protocol was repeated three times for each mouse during all individual NCS experiments. Amplitude was chosen as the principal variable in the data analysis because it was the best predictor of the physiologic changes at the muscle motor unit level.

Sciatic Functional Index (sfi) And Walking Track

After injection of BoNT-A and saline, the walking track of each group (6 mice per group) was observed weekly for 6 weeks and analyzed to calculate the SFI. A 10 cm × 10 cm × 24 cm box was made and a piece of paper of the same length and width as the box was placed under it. The hind feet of the mice were painted with ink, and the mice were placed at the right end of the box. Then, by tapping the box, the mice were forced to move to the left end of the box. The footprints of the mice were marked on the paper. The following three indicators were measured for both the damaged foot (E) and the normal foot (N): (1) print length (PL), which is the distance from the heel to the third toe; (2) the distance of the toe spread (TS), which is the distance from the first to the fifth toe; and (3) intermediary toe spread (IT), which is the distance from the second to the fourth toe.

SFI was measured using the Bain-Mackinnon-Hunter (BMH) sciatic functional index formula: SFI = -38.3*(EPL-NPL)/NPL + 109.5*(ETS-NTS)/NTS + 13.3*(EITS-NITS)/NITS − 8.8. An SFI = 0 shows normalcy, while SFI = -100 indicates serious nerve damage. The SFIs of four mice per group were measured from week 1 to 5 and three mice per group in the final week.

Muscle Mass And Volume

The mice (6 mice per group) were sacrificed every week for 6 weeks, and after peeling their skin, the changes in calf muscle volume reduction were evaluated by stereoscopic microscopy (OLYMPUS, SZ2-LGB, Tokyo, Japan) and PRIMOS^{LITE} (GFMesstechnik GmbH, Berlin, Germany). The volume measurement result refers to the following: PRIMOS^{LITE} software (PRIMOS^{LITE} version 5.8E) was used for analysis of the degree to which parallel projection stripes transmitted on the gastrocnemius mass was changed by the height difference of the gastrocnemius mass. The value refers to the volume. The average of three volume measurements taken from one mouse was used.

Protein Extraction And Western Blot Analysis

The total protein content of the gastrocnemius muscle tissue samples (3 mice per group) was homogenized using a homogenizer (TissueLyser , QIAGEN, Tokyo, Japan) in RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% Na deoxycholate, Triton X-100, and protease inhibitors). Tissue homogenates were incubated on ice for 15 min, centrifuged (GYROZEN, 1730MR, Korea) at 18,000 g for 20 min at 4 °C and the supernatants were collected. The protein concentration was subsequently quantified using a BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Twenty milligrams of protein from each sample was separated by 12% polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane (Immobilon, Millipore, Bedford, MA, USA). The membrane was saturated with 5% skim milk in Tris-buffered saline containing 0.5% Tween 20. Western blot analysis was performed by first incubating the membrane in antibodies against transforming growth factor (TGF)-β1 (ab2486, Abcam, Cambridge, UK), SNAP-25 (sc-7539, Santa Cruz Biotechnology, Santa Cruz, CA, USA),

brain-derived neurotrophic factor (BDNF) (sc-546, Santa Cruz Biotechnology), and β-actin (sc-1616, Santa Cruz Biotechnology) at 4℃ for 12 h, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Vector Labs, Inc., Burlingame, CA, USA) at room temperature for 1 h. Bound antibodies were detected using a SuperSignal™ West Pico Chemiluminescent Substrate (PIERCE Biotechnology Inc., Rockford, IL, USA) and assessed using a ChemiDoc™ XRS + System (Bio-RAD, Hercules, CA, USA).

Histological Analysis

Muscle tissue biopsy specimens (3 mice per group) were collected, immediately fixed with 10% paraformaldehyde in PBS and incubated overnight at 4℃. The samples were dehydrated, embedded in paraffin wax, and cleaved with a microtome into 5-μm serial transverse sections. The sections were then transferred to treated slides (Thermo Fisher Scientific, Pittsburgh, PA, USA), deparaffinized, and stained with hematoxylin and eosin (DAKO, Carpinteria, CA, USA) or Trichrome Stain Kit (Modified Masson's; ScyTek Laboratories, Inc., Logan, UT, USA). For immunohistochemical analysis, paraffin-embedded tissues were deparaffinized, rehydrated, and subjected to antigen retrieval using Trilogy (1:20, 920P-06-RUO, CELL MARQUE, California, USA). The sections were treated with 3% H_2O_2 solution for 30 min at room temperature to halt any endogenous peroxidase activity. After blocking nonspecific proteins in 10% normal serum with 1% bovine serum albumin (BSA) in PBST (0.1% Tween-20), the slides were incubated with antibodies against α-smooth muscle actin (SMA) (1:500, ab5694, Abcam), TGF-β1 (1:500, ab2486, Abcam), or CD34 (1:500, BD553731, BD Biosciences, Heidelberg, Germany). After PBST washing, the slides were incubated with FITC-conjugated goat-anti-rabbit IgG (1:1000, sc-2012, Santa Cruz Biotechnology). The slides were washed, incubated with the biotinylated secondary antibody at room temperature, and stained with diaminobenzidine (DAB Plus Substrate System Kit, Thermo Scientific, Fremont, CA, USA). After counterstaining with Harris hematoxylin counterstain (Sigma, St. Louis, MO, USA), the sections were dehydrated and mounted on slides with mounting solution. All stained sections were then examined by light microscopy (DM750, Leica, Wetzlar, Germany) to assess the histological changes. Data were analyzed using ImageJ 1.38 software (NIH, MD, USA). For the immunofluorescence assay, the same process used for immunohistochemical analysis was executed up until the step of incubation with the primary antibody. Briefly, gastrocnemius muscle tissue was incubated with primary antibodies to BDNF (1:200, sc-546, Santa Cruz Biotechnology) overnight at 4℃, followed by further incubation with anti-FITC-IgG at 37℃ for 1 h. Slides were washed with 1X TBS buffer and mounted in fluorescent mounting medium with DAPI (Golden Bridge International Inc., Mukilteo, WA, USA). Fluorescent images were acquired using a confocal microscope (Leica DMI400; Leica, Wetzlar, Germany).

Statistical analysis

All quantitative data are presented as the mean ± standard deviation (SD) for three independent experiments. Statistical analyses were performed using SPSS software (SPSS Inc., Chicago, IL, USA) program. Analysis of variance (ANOVA) was used for multiple comparisons. The significance of

differences between two groups was evaluated by a paired t-test. Significant values were *$p < 0.05$ and **$p < 0.01$.

Results

Treadmill exercise leads to nerve functional recovery but not change calf muscle mass and volume after BoNT-A injection

No difference in body weight was identified between the BC and BT groups during the 6 weeks. (Supplementary Fig. 1A). In addition, the muscle weight loss of the injected area continued for 5 weeks in both BC and BT groups. No significant difference ($p > 0.05$) was found in gastrocnemius muscle weight (% body weight) between BC and BT groups (Supplementary Fig. 1B). Furthermore, not only the weight difference but also the volume difference ($p > 0.05$) measured by PRIMOS and stereoscopic microscope revealed the same result (Supplementary Fig. 1C–E).

To confirm the efficacy of the nerve blocking induced by BoNT-A in this experiment, NCS was used. Three days after BoNT-A injection, the amplitude value fell to 0, which indicated flaccid paralysis; the symptoms persisted for 21 days but started to rise on the 28th day (Fig. 1A, B). Also, the amplitude value of the BT group increased slightly compared with that of the BC group as a result of treadmill exercise ($p < 0.05$). This result indicated that neural transmission was equally blocked by BoNT-A injection in both groups but neuronal reinnervation by treadmill exercise did not occur. BoNT-A-administered mice were unable to stand on the plantar surface of their toes. SFI is widely used as a measure of peripheral nerve damage. In this study, SFI was used to assess the effect of exercise on the functional recovery of the nerves. After BoNT-A injection, the SFI value of the two groups (BC and BT) decreased to -100 with no differences (Fig. 1C). SFI values of BoNT-A-treated groups (BC and BT) were significantly lower than those of BoNT-A non-treated groups on day 2. SFI values were significantly higher in the BT group than those in the BC group from day 7 to 28 ($p < 0.05$). The results indicated that treadmill exercise improves SFI values that were decreased as a result of BoNT-A injection.

Effect of treadmill exercise on inhibition of gastrocnemius muscle atrophy and adipocyte accumulation induced by BoNT-A

The histological changes caused by BoNT-A injection are shown in Fig. 2A. Adipocyte accumulation increased in the BC group in weeks 4 and 6. However, the histopathological change was significantly lower in the BT group compared with the BC group ($p < 0.05$), as shown in Fig. 2C. These results indicate that although muscle atrophy is induced by BoNT-A, exercise prevents the accumulation of adipocyte induced by BoNT-A.

Moreover, CD34 is known to play an important role in regulating the early stage activity of satellite cells[14]. The expression of CD34 increased more in the BT group than in the BC group ($p < 0.05$) (Fig. 2B). This result implies that exercise after BoNT-A injection affects the recovery of muscle associated with satellite cells.

Effect Of Treadmill Exercise On Inhibition Of Muscle Fibrosis

To elucidate the effects of treadmill exercise after BoNT-A injection on muscle fibrosis, we conducted immunohistochemical staining with α-SMA and Masson's Trichrome staining to analyze fibrotic composition and an increase in the interstitium. The expression of collagen and α-SMA in the BC group increased compared with that in the control group, while the expression of collagen and α-SMA in the BT group was reduced compared with that in the control group (Fig. 3A–B). In addition, it is well known that expression of α-SMA is increased by TGF-β1 in the muscle fibrosis mechanism[15]. We confirmed the expression of TGF-β1 in dissolved gastrocnemius muscle (Fig. 3C–E). The expression of TGF-β1 in the BT group was significantly lower than that in the BC group ($p < 0.05$). These results suggest that BoNT-A induces muscle fibrosis, and exercise can prevent BoNT-A-induced muscle fibrosis.

Effect Of Treadmill Exercise On Bdnf Expression

Intense exercise increases BDNF levels in humans[16]. We examined whether treadmill exercise increased BDNF expression after BoNT-A injection in the gastrocnemius muscles. As a result of immunofluorescence with BDNF in the gastrocnemius muscles of groups BT and BC in week 6, BDNF expression was found to have increased more in the BT group than in the BC group ($p < 0.05$) (Fig. 4A–D). Also, the expression of SNAP-25 protein increased ($p < 0.05$) (Fig. 4A–B). These results suggest that treadmill exercise after BoNT-A treatment increases BDNF and SNAP-25 expression.

Discussion

BoNT-A acts selectively on neuromuscular junctions to inhibit the fusion of synaptic vesicles at the nerve terminal and blocks the expression of neurotransmitters, which results in the inhibition of muscle contraction[17]. The inhibition of muscle movement reduces the volume of individual cells and intercellular material that make up the muscle. In contrast, exercise generally inhibits functional degeneration of muscles, prevents neurotransmission blockers, and changes muscle protein breakdown (MPB) and MPS – resulting in the balance and growth of skeletal muscle[8]. It is known that exercise maintains muscle function by preventing replacement of muscle fibers with adipocytes and muscle fibrosis[9].

BoNT-A injections block neurotransmission, leading to functional degeneration of muscles, which then results in muscle atrophy and reduction of muscle weight[18]. Studies have shown that exercise plays a positive role in neurotransmission recovery[6, 19]. According to studies related to muscular weight and degree of nerve distribution, muscle weight is higher in muscles with regenerated nerves than in denervated muscles[20]. However, muscle weight or volume was not changed in with or without treadmill exercise. It is known that patients with Duchenne muscular dystrophy with impaired muscle utilization show increased levels of adipocyte and fibrous connective tissues[21]. It is estimated that muscle

paralysis caused by BoNT-A induced adipocytes accumulation and an increase in muscle size by exercise was prevented.

NCS and SFI are widely used as parameters to evaluate nerve function recovery[22, 23]. We demonstrated that neurotransmission was blocked for 3 days after BoNT-A injection. In addition, we identified that exercise can increase the functional recovery of motor neurons, treadmill exercise is effective in nerve function recovery, and physical activity stimulates neuroprotection.

Our study showed that exercise inhibited the accumulation of adipocytes caused by BoNT-A. BoNT-A induces adipocyte accumulation by causing neurotransmission blockage, which inhibits muscle function[24].Conversely, exercise is known to increase the quality of muscles by significantly reducing adipocyte accumulation[25]. Also, muscle fibrosis is a typical factor that reduces muscle quality. Several studies regarding the molecular mechanisms of tissue fibrosis development have shown that TGF-β1 is an important regulator and that fibroblasts that are activated by TGF-β1 show enhanced α-SMA expression[15]. In this study, BoNT-A caused muscle fibrosis by muscle paralysis following neurotransmission blockage, while exercise inhibited α-SMA expression through TGF-β1 signaling inhibition. In addition, in previous studies, satellite cells have been used to confirm skeletal muscle regeneration and are important factors in the functional recovery of muscles[26]. These satellite cells are present in the crevices on the surface of muscle fibers beneath the raised basal plate and are activated when muscle regeneration is needed[27]. CD34 is a protein that is expressed when satellite cells are activated[14]. The results showed that CD34 expression increased in the crevices of the muscle fibers after BoNT-A injection in the treadmill exercise group indicating that exercise is effective in skeletal muscle regeneration after neurotransmission blockage.

BoNT-A breaks down SNAP-25 protein, preventing the docking of acetylcholine vesicles to the cell membrane, thus blocking neurotransmission[28]. However, exercise increases SNAP-25 protein expression[29]. It promotes the release of BDNF protein, which is known to regulate neurodevelopment[30]. Consistently, the expression of SNAP-25 protein increased in the BT group. Similarly, the expression of BDNF protein increased in the BT group. This suggests that exercise increases the recovery of SNAP-25 protein expression after BoNT-A injection, supporting the SFI and NCS results which indicate that exercise has a positive effect on motor neurotransmission recovery.

The experiments have some clinical implications. In the cosmetic field, BoNT-A injections are used to induce muscular atrophy in order to reduce muscle size[5, 31]. The results showed that there was no difference in gastrocnemius muscle volume and weight with or without aerobic exercise through a treadmill for 6 weeks after BoNT-A injection. These results suggest that for BoNT-A gastrocnemius muscle injections for cosmetic purposes, the continuous movement of skeletal muscle such as that during exercise does not affect the muscle volume. Rather, exercise after BoNT-A injection may have a positive effect on functional recovery by preventing adipocyte accumulation and muscle fibrosis. The limitations of our study were that we focused on the period of the induced effect of BoNT-A-blocked

neurotransmission. Further studies will be needed to confirm changes in muscle volume due to exercise during the period in which the neurotransmitter blocking effect of BoNT-A is released.

Conclusions

In this study, we observed various aspects to confirm changes in muscle weight, neurotransmission, and functional aspects of muscle, such as preventing muscle fibrosis and adipocyte accumulation, when exercising through a treadmill after BoNT-A injection. Moreover, we identified at least two factors that regulated exercise process of muscle and nerve functional recovery, decreased TGF-β1 signaling and increased BDNF expression. Thus, the treadmill exercise prevented the progression of BoNT-A-induced muscle fibrosis and was effective in blocking neurotransmission, leading to the recovery of muscular function.

Abbreviations

BoNT-A:botulinum toxin type A, SFI:sciatic functional index, NCS:nerve conduction study, MPS:muscle protein synthesis, CMAP:compound muscle action potential, SNAP-25:synaptosomal nerve-associated protein 25, BC:BoNT-A control, BT:BoNT-A treadmill, TGF-β1:transforming growth factor-beta1; BDNF:brain-derived neurotrophic factor, α-SMA:smooth muscle actin, DAB:diaminobenzidine

Declarations

Ethics approval and consent to participate

All animal procedures were approved by the Institutional Animal Care and Use Committee of Chung-Ang University (201700028) and confirmed to all applicable National Institutes of Health guidelines.

Consent for publication

Not applicable

Availability of data and materials

The data used to support the findings of this study are available from the corresponding author upon request.

Competing interests.

The authors declare that they have no competing interests

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Author contributions

BJK and JN contributed to the conception of the study. EL and SII designed the experiment. EL, SII, and YK performed the experiment. YK and SYK analyzed the data. EL and SII drafted the manuscript. BJK and JN revised the manuscript. All authors have read and approved the manuscript

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Figures

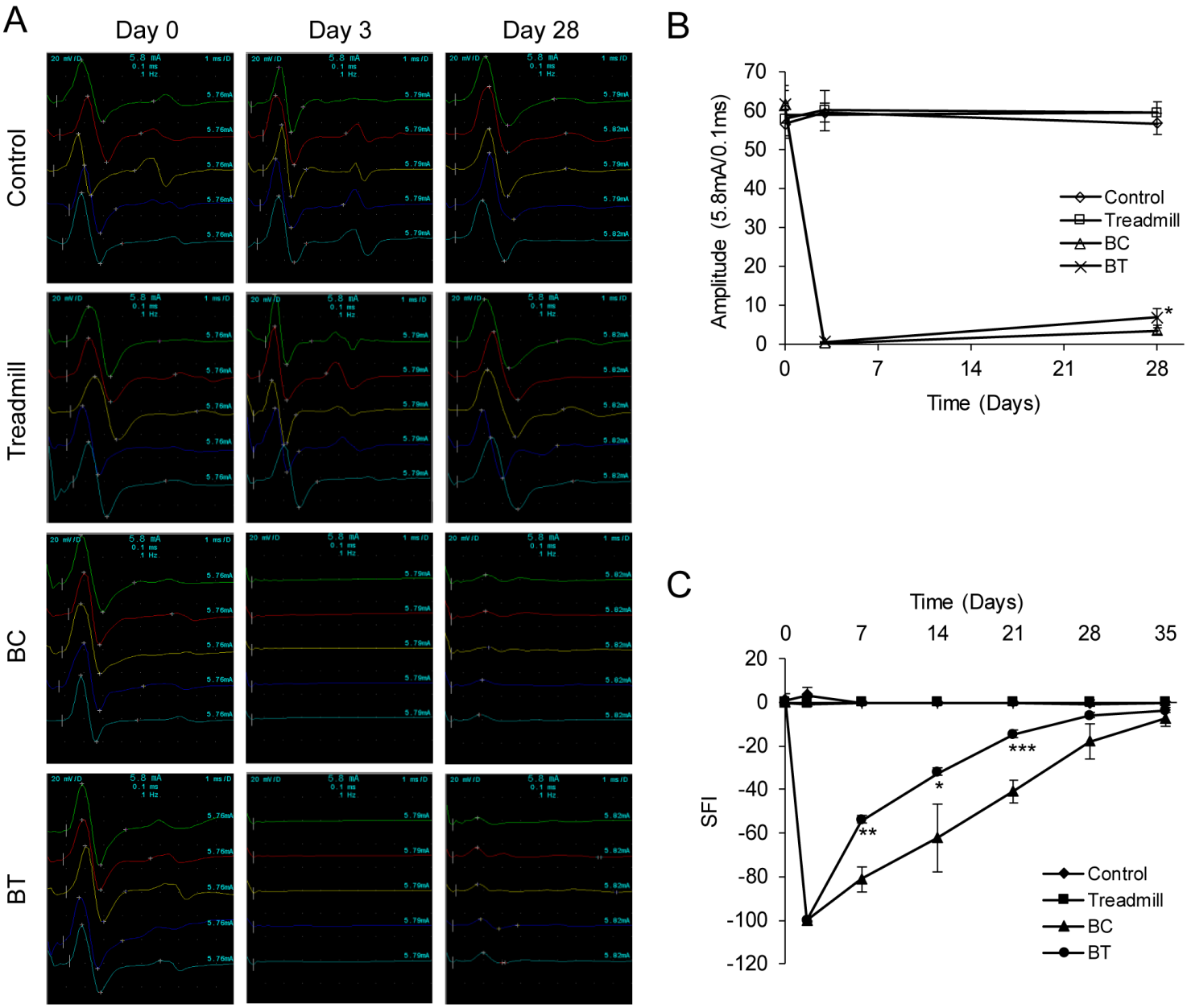

Figure 1

Effect of treadmill exercise on nerve functional recovery after BoNT-A injection. (A) Nerve conduction study was recorded before BoNT-A injection and after BoNT-A injection on day 0, 3, and 28. n=5/group. (B) A graph showing amplifications of each group. Although not statistically significant, differences between BC and BT groups were found. (C) Sciatic functional index (SFI) was measured for 35 days in four groups (BoNT-A was injected on day 2). All data represent mean ± S.D. Significant values were * P<0.05, **P<0.01, and ***P<0.001.

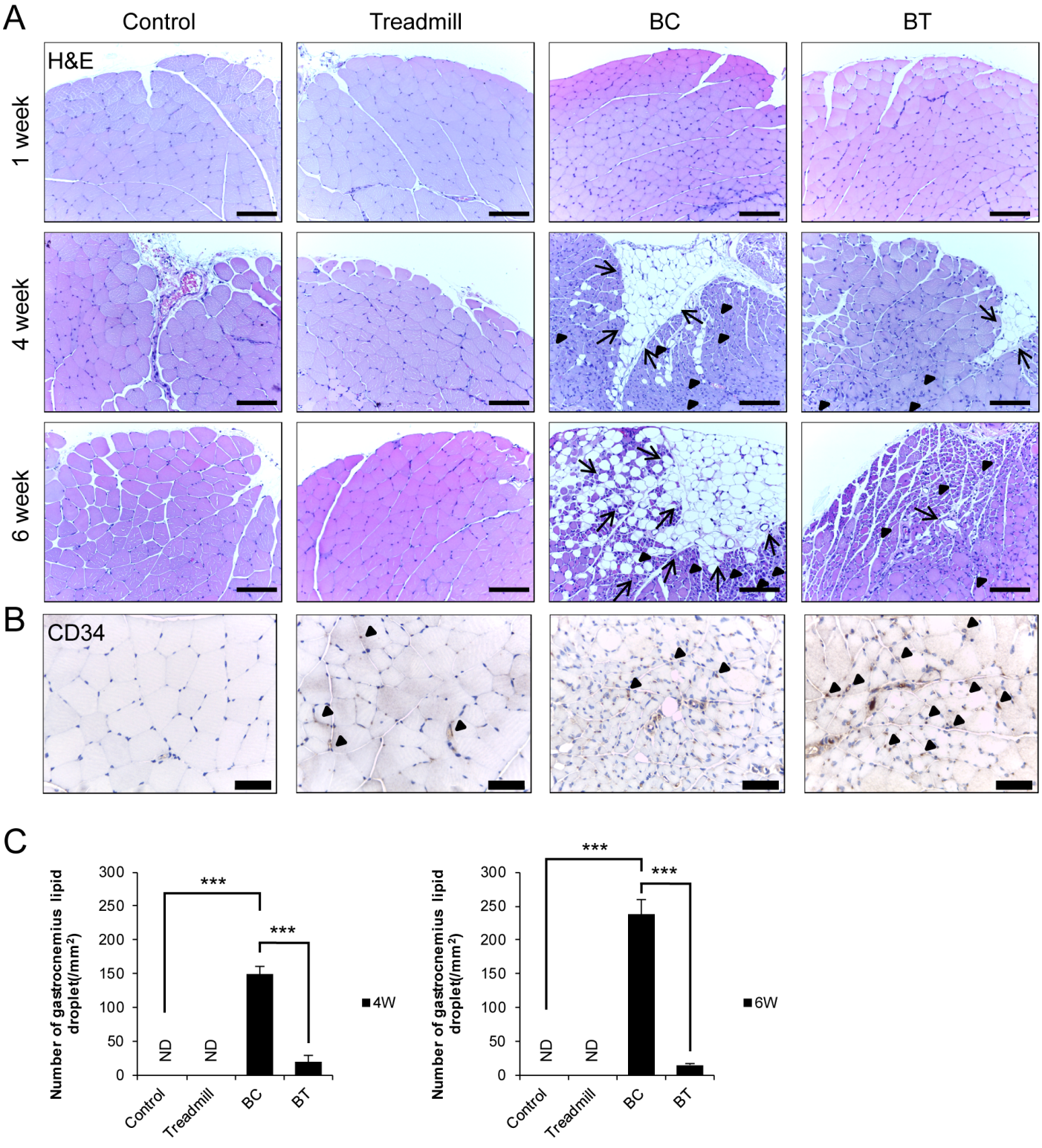

Figure 2

Effect of treadmill exercise on gastrocnemius muscle atrophy and adipocyte accumulation induced by BoNT-A. (A) Atrophy and adipocyte accumulation of gastrocnemius muscles were analyzed using hematoxylin and eosin staining during the experimental period. The black arrows indicate accumulation of adipocytes and arrowheads indicate sites of muscle atrophy. Scale bar = 50 μm. (B) The anti-CD34 antibody was stained in the gastrocnemius muscle at week 6. Arrowheads indicate expression of CD34 in

satellite cells located in the interstitium. (C) The number of gastrocnemius muscle lipid droplets decreased remarkably in the BT group compared with the BC group at the fourth and sixth weeks. All data represent mean ± S.D. Significant values were *** P<0.001. ND: Not detected.

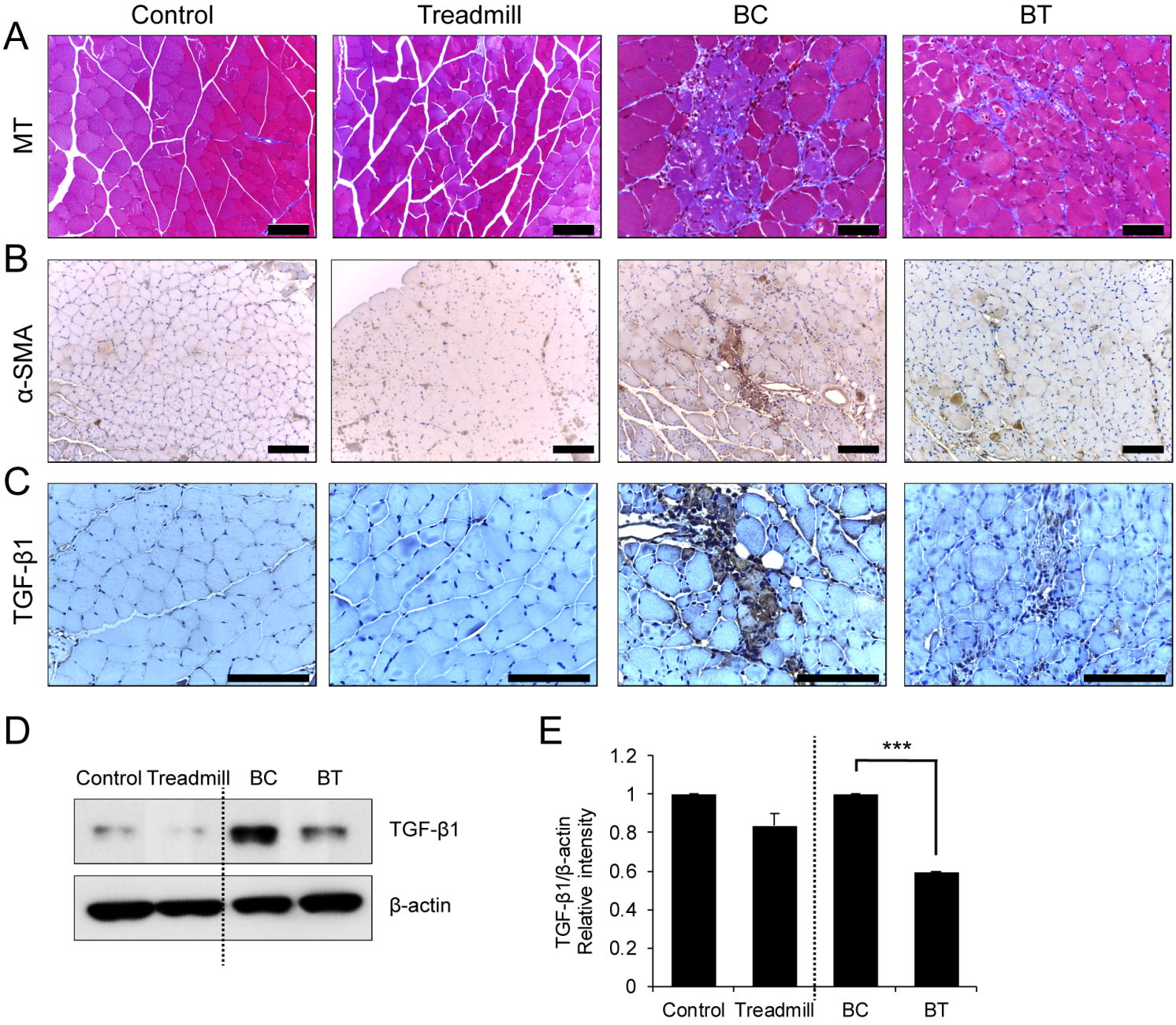

Figure 3

Effect of treadmill exercise on inhibition of muscle fibrosis. Gastrocnemius muscle fibrosis was confirmed using Masson's Trichrome Stain (A) and immunohistochemical staining with anti-α-SMA. (B) at week 6. Increase in protein (collagen and TGF-β1) by BoNT-A injection was reduced by treadmill exercise. (C) For immunohistochemical staining with anti-TGF-β1 expression in the gastrocnemius muscle, slides were stained at week 6. (D, relative E) Western blot analysis of anti-TGF-β1 in gastrocnemius muscle at week 6. The BT group exhibited significantly less TGF-β1 than the BC group. β-actin was used as loading control. All data represent mean ± S.D. Significant values were *** P<0.001.

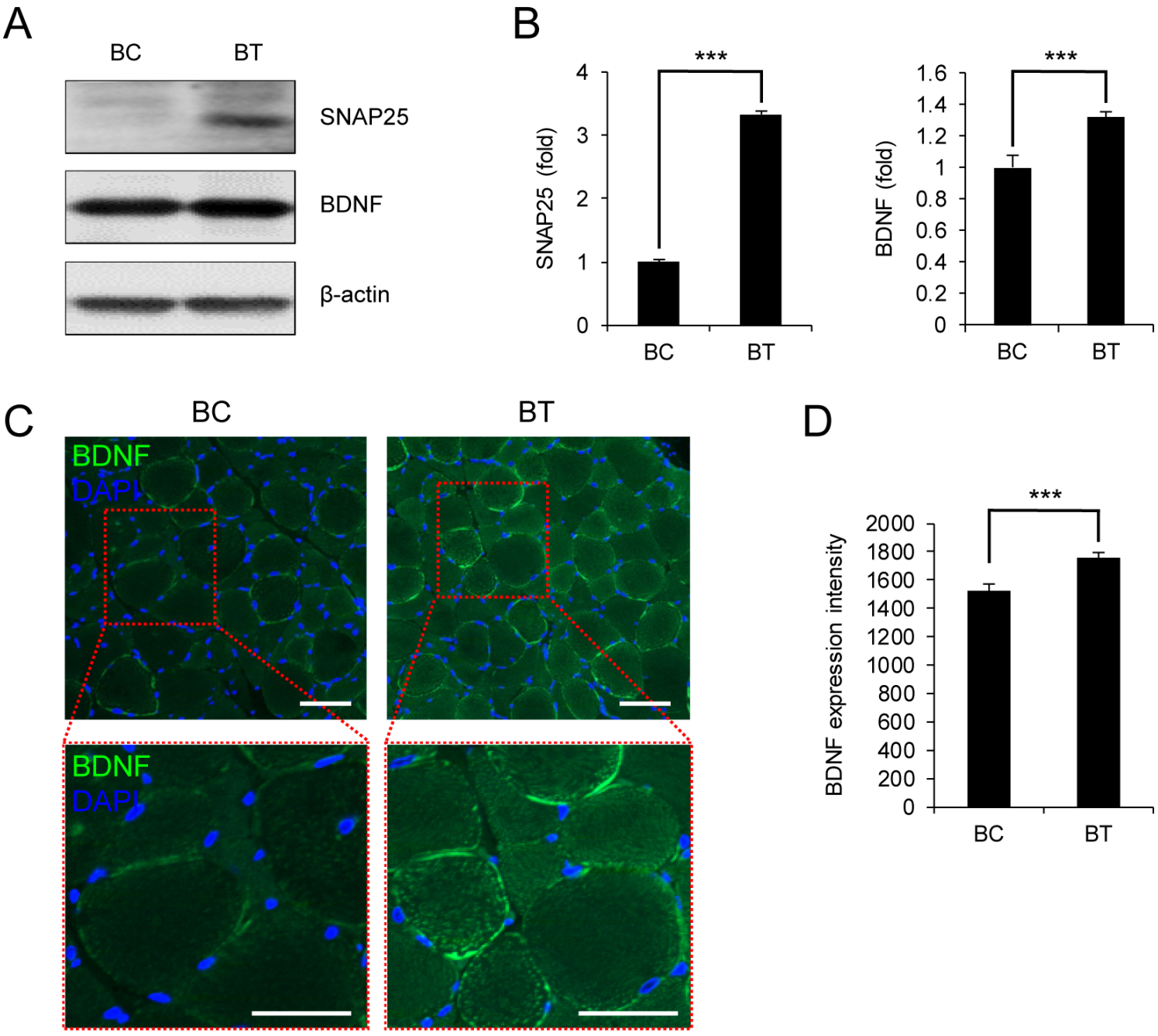

Figure 4

Effect of treadmill exercise on SNAP-25 and BDNF expression. (A) Representative images of Western blot assay for SNAP-25 and BDNF proteins in muscle tissue. (B) Bar graph, intensities of immune-reactive bands on Western blot were quantified by densitometric analysis. (C) Representative confocal images show BDNF expression in the gastrocnemius muscle of two groups (BC and BT) at week 6. (D) The BDNF expression intensity is quantitated. All data represent mean ± S.D. Significant values were *** P<0.001.

Supplementary Files

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- NC3RsARRIVEGuidelinesChecklistfillable.pdf
- SupplementaryMaterial.pdf