Exercise training improves adipocyte accumulation and muscle fibrosis by TGF- β 1 and α -SMA reduction after botulinum toxin type A administration in mice

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Abstract

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

Methods: After the injection of 0.5 units of BoNT-A into the gastrocnemius muscle of ICR mice, treadmill exercise was conducted for a 6-week period, after which the muscle volume, weight, and sciatic functional index (SFI) was determined and a nerve conduction study (NCS) and histological evaluation were performed.

Results: After exercise, there was no change in the gastrocnemius weight and volume, but NCS and SFI increased. Exercise prevented induced adipocyte accumulation and muscle fibrosis. Moreover, transforming growth factor- β 1 and α -smooth muscle actin expressions decreased and CD34, brain-derived neurotrophic factor, and synaptosomal nerve-associated protein 25 expressions increased when treadmill exercise was performed after BoNT-A administration.

Conclusions: Exercise can effectively recover the nerve function and would aid in muscle function recovery by preventing fat cell accumulation and muscle fibrosis after BoNT-A administration.

Background

Botulinum toxin type A (BoNT-A) is a neurotoxin that selectively acts on the neuromuscular junction [1]. BoNT-A cleaves the synaptosomal nerve-associated protein 25 (SNAP-25) protein, which helps in exocytosis of vesicles containing neurotransmitters at the nerve endings [2]. Thus, blocking of neurotransmitters by BoNT-A through the prevention of synaptic vesicle fusion leads to muscle paralysis[2]. The effect of BoNT-A lasts for about 6 months after its injection, at which point, muscle mass, muscle contraction, and nerve function are restored[3]. BoNT-A is also widely used in dermatology for cosmetic purposes to reduce muscle size [4].

Exercise inhibits the degeneration of muscular function and improves neural function [5]. It also affects the growth of skeletal muscle by increasing muscle protein synthesis (MPS) and enhancing muscle function by preventing the replacement of muscle fibers with adipocytes and fibrosis[6, 7]. Numerous studies proved that exercise increases muscle function[8]. Various experimental models of the effect of exercise after botulinum toxin administration have been reported. In a rat exercise training model, 7 days of voluntary wheel running increased the mass and fiber size of BoNT-A–injected muscle without consistently changing muscle production or number in juvenile rats[9]. 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 such as *TTN* and *ANKRD2* and muscle LIM protein in BoNT-A–injected muscle[10]. Another model on the effect of electrical stimulation (ES) exercise on muscle-injected BoNT-A has been reported [11]. ES exercise partially prevented muscle weakness, atrophy, and contractile material loss in the injected muscles and mostly prevented degeneration of the contralateral, non-injected muscles.

However, the effects of exercise on the muscles and nerves after BoNT-A administration have not been clearly demonstrated. Treadmill is used in animal experiments to exert aerobic exercise effects[12]. In a study of peripheral nerve injury and recovery, treadmill exercise improved nerve and muscle response [13-16]. In this study, we investigated the effect of treadmill exercise on muscle weight and functional recovery when sustained contraction and relaxation were induced by treadmill exercise after nerve transmission blockage by BoNT-A.

Methods

Animals and experimental design

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 was used for euthanasia. CO₂ gas was gradually filled at 10% to 30% for the chamber volume per min [17]. The mice were separated into four groups:

- (1) Control group – saline injection, no exercise
- (2) Treadmill group – saline injection, treadmill exercise
- (3) BoNT-A control (BC) group – BoNT-A injection, no exercise
- (4) BoNT-A treadmill (BT) group – BoNT-A injection, treadmill exercise

For the control and saline groups, 10 µL of saline was injected into the right gastrocnemius muscle of each mouse, while 0.5 unit 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 conformed 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 each) for 5 min of running at 50 m/min on a 45-cm treadmill belt to ensure that all mice performed similar treadmill work before BoNT-A administration. A JD-A-09 treadmill manufactured by JEUNGDO Bio & Plant Co., Ltd. (Seoul, Korea) was used in this study. The 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. The treadmill exercise was performed for 20 min at a speed of 15 m/min and temperature of 10°C five times a week over a 6-week period.

Nerve conduction study (NCS): Electrophysiology

The Dantec™ Keypoint® Focus (Natus Neurology, Middleton, WI, USA) instrument was used to record nerve conduction of the gastrocnemius muscle (5 mice per group); 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 received supramaximal electric stimulation at the same position each time. The compound muscle action potential amplitude (mV) (peak to peak), distal latency (ms), and area (mm²) were recorded each time. The protocol was repeated three times for each mouse during individual nerve conduction study (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 sciatic functional index (SFI). A 10 cm × 10 cm × 24 cm box was made and a piece of paper of the corresponding length and width 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. The box was then tapped and 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 the damaged (E) and normal (N) foot: (1) print length, the distance from the heel to the third toe; (2) toe spread, the distance from the first to the fifth toe; and (3) intermediary toe spread (IT), the distance from the second to the fourth toe.

SFI was measured using the Bain-Mackinnon-Hunter SFI formula: $SFI = -38.3 \cdot (EPL - NPL) / NPL + 109.5 \cdot (ETS - NTS) / NTS + 13.3 \cdot (EITS - NITS) / NITS - 8.8$. An SFI = 0 is normal, while an SFI = -100 indicates serious nerve damage. The SFI values of four mice per group were measured in weeks 1–5, while those of three mice per group were measured in the final week.

Muscle mass and volume

The mice (3 mice per group) were euthanized every week for 6 weeks; after their skin was removed, changes in calf muscle volume reduction were evaluated using stereoscopic microscopy (OLYMPUS, SZ2-LGB, Tokyo, Japan) and PRIMOS^{LITE} (GFMeasstechnik GmbH, Berlin, Germany). The volume measurement result refers to the following: PRIMOS^{LITE} software (PRIMOS^{LITE} version 5.8E) was used to analyze 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 (three mice per group) was homogenized using a homogenizer (TissueLyser $\square$; QIAGEN, Tokyo, Japan) in RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 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 $\times g$ for 20 min at 4°C, and supernatants were collected. The protein concentration was subsequently quantified using the 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 blotting was performed by first incubating the membrane in antibodies against transforming growth factor (TGF)- β 1 (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°C 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 the 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 (three mice per group) were collected, immediately fixed in 10% paraformaldehyde in phosphate buffered saline (PBS), and incubated overnight at 4°C. The samples were dehydrated, embedded in paraffin wax, and cleaved with a microtome into 5- μ m 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 the 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, Rocklin, CA, USA). The sections were treated with 3% H₂O₂ solution for 30 min at room temperature to halt any endogenous peroxidase activity. After nonspecific proteins were blocked in 10% normal serum with 1% bovine serum albumin in PBS with 0.1% Tween-20 (PBST), the slides were incubated with antibodies against α -smooth muscle actin (SMA) (1:500; ab5694; Abcam), TGF- β 1 (1:500; ab2486; Abcam), or CD34 (1:500; BD553731; BD Biosciences, Heidelberg, Germany). After being washed in PBST, the slides were incubated with

fluorescein isothiocyanate (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 using mounting solution. All stained sections were then examined using light microscopy (DM750; Leica, Wetzlar, Germany) to assess histological changes. Data were analyzed using ImageJ 1.38 software (NIH, Bethesda, 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, the gastrocnemius muscle tissue was incubated with primary antibodies to BDNF (1:200; sc-546; Santa Cruz Biotechnology) overnight at 4°C, followed by further incubation with anti-FITC-IgG at 37°C for 1 h. The slides were washed with 1× Tris-buffered saline and mounted in fluorescent mounting medium with DAPI (Golden Bridge International Inc., Mukilteo, WA, USA). Fluorescent images were acquired using a confocal microscope (DMI400; Leica).

Statistical analysis

All quantitative data are presented as mean $\pm$ standard deviation (SD) for three independent experiments. Statistical analyses were performed using SPSS software (SPSS Inc., Chicago, IL, USA). For multiple comparisons, one-way analysis of variance was used with post hoc Bonferroni test. We considered P values <0.05 statistically significant. Significance levels are designated as follows: * $p < 0.05$; ** $p < 0.01$.

Results

Treadmill exercise leads to nerve functional recovery but not change in 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-week study period ($p > 0.05$) (Supplementary Figure. 1A). In addition, the muscle weight loss of the injected area continued for 5 weeks in both groups. No significant difference ($p > 0.05$) was found in gastrocnemius muscle weight (% body weight) between the groups (Supplementary Figure. 1B). Furthermore, not only the difference in weight but also the difference in volume ($p > 0.05$) measured using PRIMOS and stereoscopic microscope were similar between groups (Supplementary Figures. 1C–1E).

NCS was used to confirm the efficacy of the BoNT-A–induced nerve block in this experiment. Three days after BoNT-A administration, the amplitude value fell to 0, which indicated flaccid paralysis; the symptoms persisted for 21 days, but the value started to rise on the 28th day (Figures 1A, 1B). 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 administration in both groups but neuronal reinnervation was not induced by the treadmill exercise. BoNT-

A-injected mice were unable to stand on the plantar surfaces 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 functional nerve recovery. After BoNT-A administration, the SFI value of the two groups decreased to -100 with no differences ($p>0.05$) (Figure 1C). SFI values of the BoNT-A-treated groups (BC and BT) were significantly lower than those of BoNT-A-non-treated groups on day 2 ($p<0.05$). SFI values were significantly higher in the BT group than 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 administration.

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

Histological changes caused by BoNT-A injection are shown in Figure 2A and Supplementary Figure 2. Adipocyte accumulation increased in the BC group in weeks 4 and 6. However, the histopathological change was significantly lower in the BT group than in the BC group ($p<0.05$) (Figure 2C). These results indicate that although muscle atrophy is induced by BoNT-A, exercise prevents the accumulation of adipocytes otherwise induced by BoNT-A.

Moreover, CD34 plays an important role in regulating the early-stage activity of satellite cells[18]. CD34 expression increased more in the BT group than in the BC group ($p<0.05$) (Figure 2B and Supplementary Figure 2D). This result implies that exercise after BoNT-A injection affects satellite cell-associated muscle recovery.

Effect of treadmill exercise on muscle fibrosis

To elucidate the effects of treadmill exercise on muscle fibrosis after BoNT-A injection, we conducted immunohistochemical staining with α -SMA and Masson's trichrome staining to analyze fibrotic composition and detect an increase in the interstitium. Collagen and α -SMA expressions were increased in the BC group ($p<0.05$) compared to those in the control group, whereas reduced in the BT group ($p<0.05$) compared to the BC group (Figures 3A, 3B and Supplementary Figure 3A, 3B). In addition, α -SMA expression is increased by TGF- β 1 in the muscle fibrosis mechanism[19]. We confirmed TGF- β 1 expression in the dissolved gastrocnemius muscle (Figures 3C–3E and Supplementary Figure 3C). TGF- β 1 expression was significantly lower in the BT group than in the BC group ($p < 0.05$). These results suggest that BoNT-A induces muscle fibrosis, which can be prevented by exercise.

Effect of treadmill exercise on BDNF expression

Intense exercise increases BDNF levels in humans[20]. We examined whether treadmill exercise increased BDNF expression after BoNT-A administration in the mice gastrocnemius muscles. As a result of immunofluorescence with BDNF in the gastrocnemius muscles of the BT and BC groups in week 6, BDNF expression increased more in the BT group than in the BC group ($p < 0.05$) (Figures 4A–4D, Supplementary Figure 4). SNAP-25 expression increased ($p < 0.05$) (Figures 4A–4B). These results suggest that treadmill exercise after BoNT-A treatment increases BDNF and SNAP-25 expressions.

Discussion

BoNT-A selectively acts on neuromuscular junctions to inhibit the fusion of synaptic vesicles at the nerve terminal and blocks the expression of neurotransmitters, resulting in the inhibition of muscle contraction[21]. The inhibition of muscle movement reduces the volume of individual cells and intercellular material that comprise the muscle. In contrast, exercise generally inhibits functional degeneration of muscles, prevents neurotransmission blockers, and changes muscle protein breakdown and MPS, resulting in the balance and growth of skeletal muscle[6]. Exercise maintains muscle function by preventing the replacement of muscle fibers with adipocytes and muscle fibrosis[7].

BoNT-A administration blocks neurotransmission, leading to functional muscle degeneration and resulting in muscle atrophy and reduction in muscle weight[22]. Exercise plays a positive role in neurotransmission recovery[23]. 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 [24, 25]. However, neither muscle weight nor volume was changed with or without treadmill exercise. Patients with Duchenne muscular dystrophy and impaired muscle utilization show increased proportions of adipocytes and fibrous connective tissues[26]. Muscle paralysis also caused by BoNT-A–induced adipocyte accumulation [27].

NCS and SFI are widely used to evaluate nerve function recovery [28]. We demonstrated that neurotransmission was blocked for 3 days after BoNT-A administration. We also identified that exercise can increase the functional recovery of motor neurons, treadmill exercise effectively recovered nerve function, and physical activity stimulates neuroprotection.

Our study showed that exercise inhibited BoNT-induced adipocyte accumulation. BoNT-A induces adipocyte accumulation by causing neurotransmission blockage, thereby inhibiting muscle function[29]. Conversely, exercise increases muscle quality by significantly reducing adipocyte accumulation[30]. Muscle fibrosis is a typical factor that reduces muscle quality [31]. Several studies of the molecular mechanisms of tissue fibrosis development have shown that TGF- β 1 is an important regulator and that TGF- β 1–activated fibroblasts show enhanced α -SMA expression[19]. In this study, BoNT-A caused muscle fibrosis by muscle paralysis following neurotransmission blockage, while exercise inhibited α -SMA expression through TGF- β 1 signaling inhibition. In addition, in previous studies, satellite cells have been used to confirm skeletal muscle regeneration and are important factors in muscle functional recovery[32]. These satellite cells are present in the crevices on the surface of muscle fibers beneath the raised basal

plate and activated when muscle regeneration is needed[33]. CD34 is a protein that is expressed upon satellite cell activation[18]. The results showed that CD34 expression increased in the crevices of muscle fibers after BoNT-A administration in the treadmill exercise group, indicating that exercise induces 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 [2]. However, exercise increases SNAP-25 expression[34, 35] and promotes the release of BDNF protein, which regulates neurodevelopment[36]. In this study, SNAP-25 expression consistently increased in the BT group. Similarly, BDNF expression increased in the BT group. This finding suggests that exercise increases the recovery of SNAP-25 expression after BoNT-A administration, supporting the SFI and NCS results that indicate that exercise positively affects motor neurotransmission recovery.

The findings of these experiments have some clinical implications. In the cosmetic field, BoNT-A administration is used to induce muscular atrophy to reduce muscle size [37]. The results showed no difference in gastrocnemius muscle volume and weight with or without 6 weeks of aerobic treadmill exercise after BoNT-A administration. These results suggest that for BoNT-A administration into the gastrocnemius muscle for cosmetic purposes, continuous movement of the skeletal muscle, such as that during exercise, does not affect muscle volume. Rather, exercise after BoNT-A administration may positively affect functional recovery by preventing adipocyte accumulation and muscle fibrosis. The present findings may also increase our understanding of the BoNT-A kinetics of exercise in patients, such as those with cerebral palsy. Children with cerebral palsy who receive multiple high-dose BoNT-A injections tend to have more serious side effects than those who receive a single or multiple low-dose injections[38]. Massin et al. used an incremental walking treadmill protocol to assess the effect of intramuscular BoNT-A administration on the energy cost of movement and walking endurance in 15 children with cerebral palsy aged 4–13 years. The study provided clinical evidence of the efficacy of BoNT-A at reducing the energy cost of movement and improving the endurance of the spastic muscles in children with cerebral palsy [39].

The limitation of our study was that we focused on the period of the induced effect of BoNT-A–blocked neurotransmission. Further studies are needed to confirm exercise-induced changes in muscle volume 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 characteristics such as preventing muscle fibrosis and adipocyte accumulation using treadmill exercise after BoNT-A administration. Moreover, we identified at least two factors that regulated the exercise process of muscle and nerve functional recovery, namely, decreased TGF- β 1 signaling and increased BDNF expression. Moreover, we show the treadmill exercise prevented the progression of BoNT-A–induced muscle fibrosis and effectively blocked neurotransmission, leading to muscular function

recovery. Thus, exercise may lead to enhanced treatment and recovery in patients receiving BoNT-A administration to relax the hyperactive muscles.

List Of Abbreviations

α -SMA, alpha-smooth muscle actin; BC, BoNT-A control; BDNF, brain-derived neurotrophic factor; BoNT-A, botulinum toxin type A; BT, BoNT-A treadmill; MPS, muscle protein synthesis; NCS, nerve conduction study; SFI, sciatic functional index; SNAP-25, synaptosomal nerve-associated protein 25; TGF- β 1, transforming growth factor-beta1

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 conformed 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 no competing interests.

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Authors' contributions

BJK contributed to the conception and designed of the study. JN, EL, and SII contributed to the data acquisition and analysis. YK and SYK analyzed the data. JN, EL, and SII drafted the manuscript. BJK revised the manuscript. All authors have read and approved the manuscript. All authors agreed both to be personally accountable for the author's own contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even any in which the authors were not personally involved, are appropriately investigated and resolved and the resolution documented in the literature.

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Figures

Figure 1

Effect of treadmill exercise on SNAP-25 and BDNF expression. (A) Representative Western blot assay images for synaptosomal nerve-associated protein 25 (SNAP-25) and brain-derived neurotrophic factor (BDNF) proteins within muscle tissue. (B) Bar graph, immune-reactive band intensities on Western blot quantified by densitometric analysis. (C) Representative confocal images show BDNF expression in the gastrocnemius muscle of two groups (BoNT-A control [BC] and BoNT-A treadmill [BT]) at week 6. Scale bar = 50 μ m. (D) BDNF expression intensity is quantitated. All data represent mean $\pm$ SD. ***P < 0.001.

Figure 2

Effect of treadmill exercise on muscle fibrosis. Gastrocnemius muscle fibrosis confirmed using Masson’s trichrome stain (A) and immunohistochemical staining with anti- α -smooth muscle actin (B) staining at week 6. Scale bar = 100 μ m. Increase in protein (collagen and transforming growth factor- β 1 [TGF- β 1]) by botulinum toxin type A (BoNT-A) injection reduced by the treadmill exercise. (C) For immunohistochemical

staining with anti-TGF- β 1 expression in the gastrocnemius muscle, slides were stained at week 6. Scale bar = 100 μ m. (D, relative E) Western blotting of anti-TGF- β 1 in the gastrocnemius muscle at week 6. The BoNT-A treadmill (BT) group exhibited significantly less TGF- β 1 than the BoNT-A control (BC) group. β -Actin was used as loading control. All data represent mean $\pm$ SD. *** $P < 0.001$.

Figure 3

Effect of treadmill exercise on gastrocnemius muscle atrophy and adipocyte accumulation induced by BoNT-A. (A) Atrophy and adipocyte accumulation of the gastrocnemius muscles were analyzed using hematoxylin and eosin staining. Black arrows indicate adipocyte accumulation and arrowheads indicate muscle atrophy sites. Scale bar = 100 μ m. (B) Anti-CD34 antibody stained in the gastrocnemius muscle at week 6. Arrowheads indicate CD34 expression in satellite cells within the interstitium. (C) Number of gastrocnemius muscle lipid droplets decreased remarkably in the BT versus BC group at weeks 4 and 6. All data represent mean $\pm$ SD. ***P < 0.001. ND, not detected.

Figure 4

Effect of treadmill exercise on nerve functional recovery after BoNT-A injection. (A) The nerve conduction study was recorded before and after botulinum toxin type A (BoNT-A) injection on days 0, 3, and 28; n = 5/group. (B) Graph showing amplifications of each group. Although not 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 $\pm$ SD. *P < 0.05, **P < 0.01, and ***P < 0.001.

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