Effects of Treadmill Running on Rat Gastrocnemius Function Following Botulinum Toxin A Injection

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ABSTRACT: Exercise can improve and maintain neural or muscular function, but the effects of exercise in physiological adaptation to paralysis caused by botulinum toxin A has not been well studied. Twenty-four rats were randomly assigned into control and treadmill groups. The rats assigned to the treadmill group were trained on a treadmill three times per week with the running speed set at 15 m/min. The duration of training was 20 min/session. Muscle strength, nerve conduction study and sciatic functional index (SFI) were used for functional analysis. Treadmill training improved the SFI at 2, 3, and 4 weeks ($p = 0.01$, 0.004, and 0.01, respectively). The maximal contraction force of the gastrocnemius muscle in the treadmill group was greater than in the control group ($p < 0.05$). The percentage of activated fibers was higher in the treadmill botox group than the percentage for the control botox group, which was demonstrated by differences in amplitude and area of compound muscle action potential (CMAP) under the curve between the groups ($p < 0.05$). After BoNT-A injection, treadmill improved the physiological properties of muscle contraction strength, CMAP amplitude, and the recovery of SFI. © 2011 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res

Keywords: botulinum toxin; treadmill; CMAP amplitude; nerve conduction study; muscle strength

Exercise is used to improve and maintain aerobic, neural, or muscular function.[1,2] The therapeutic effects of exercise that maintain neural or muscle function improve muscle tetanic contraction force, induce neurogenesis in the adult central nervous system, and promote functional recovery and cortex plasticity.[2–5] Treadmill exercise, a dynamic training approach, is a frequently used active physical activity model that provides therapeutic and experimental intervention for walking and gait assessment.[6,7] In studies of peripheral nerve injury and repair, treadmill training led to improvements in neuromuscular response, fatigue index, and twitch characteristics.[8] But the advantages of treadmill exercise related to the recovery process of botulinum toxin exposure are not clearly understood.

Botulinum toxin A (BoNT-A) has been widely used in the management of spasticity such as in patients with stroke or cerebral palsy.[9,10] Although several studies support the beneficial effects of treadmill training in patients with spasticity,[11,12] the effects of this training in physiological adaptation to paralysis caused by BoNT-A is still an unanswered question. BoNT-A is a bacterial zinc-dependent endopeptidase that acts specifically on neuromuscular junctions.[13] After endocytotic uptake of BoNT-A, the light chain of BoNT-A cleaves synaprosomal-associated docking protein SNAP-25,[14] which effectively denervates a muscle by inhibiting the release of acetylcholine at the neuromuscular junction (NMJ) and decreases the ability of a spastic muscle to generate force.[15] Patients with botulism typically present symptoms such as dysphagia, dysarthria, respiratory failure, and generalized weakness. The paralysis produced by BoNT-A elicits nerve sprouting, and the newly created synapses are responsible for the initial synaptic transmission following botulinum poisoning.[16] The effect of botulinum toxin lasts for ~3 to 6 months, but following BoNT-A injection, the muscles regain muscle mass and recover contraction ability.[17]

The temporal blockade of neuromuscular function is an attractive feature of BoNT-A. The blockade allowed us to investigate the modification of the muscle physiological activity from paralysis to recovery with the combination of BoNT-A and treadmill exercise. To evaluate the efficacy of the treadmill exercise, we investigated the recovery process after training by examining walking track changes, analyzing the muscle twitch contraction force, and investigating electrophysiological parameters. Our aim was to obtain physiological information about the effect of combining treadmill exercise and BoNT-A injection, which may improve our overall understanding of the mechanisms underlying the current treatments for botulism.

METHODS

Animals

Twenty-four male Sprague–Dawley rats (8 weeks of age) were housed in an animal resources facility in a room with a controlled temperature (20–22°C) and a 12 h light/dark cycle. Rat chow and water were provided ad libitum. Body weight was measured weekly. All experimental procedures were approved by the Animal Care and Use Committee at Taichung Veterans General Hospital and Chung Hsing University.

Experimental Groups

The rats were randomly assigned to two groups: untrained controls ($n = 12$, 6 for the muscle contraction force test and 6

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for the electrophysiological test) and the treadmill group ($n = 12$, 6 for the muscle contraction force test and 6 for the electrophysiological test). All rats received a surgical procedure and botulinum toxin intramuscular injection as described below. In the control group, the left gastrocnemius muscle that received a botulinum toxin injection was designated the control botox (CB) muscle, and the right gastrocnemius muscle was designated the control muscle. For the treadmill group, the left and right gastrocnemius muscles were designated the treadmill botox (TB) muscle and the treadmill muscle, respectively.

Surgery

After anesthesia was given (1.0% isoflurane), a small skin incision was made along the posterior aspect of the hind limb to expose the gastrocnemius muscle. Vials of lyophilized botulinum toxin A (BOTOX, Allergan, Irvine, CA) were reconstituted in 2 ml of normal saline solution in a 100-unit vial (50 units/ml). The BoNT-A was injected into the medial and lateral heads of the left gastrocnemius muscle (equally in the left and right belly) at a dosage of 3 units/kg bodyweight using a Hamilton syringe (Fischer Scientific, Pittsburgh, PA). An equivalent volume of saline was injected into the right gastrocnemius muscle to serve as a contralateral control. The animals were euthanized 4 weeks after BoNT-A injection, and the gastrocnemius muscles were harvested from both hind limbs.

Treadmill Exercise Protocol

Three days after surgery and intramuscular BoNT-A injection, the rats assigned to the treadmill group were trained on a motorized treadmill with a 10° slope. Training consisted of running on the treadmill 3 times/week for 4 weeks. The running speed was set at 15 m/min, and the duration of training was 20 min/session. The rats were forced to run with an electric shock device.

Functional Assessment

Changes in gait pattern were assessed with the sciatic functional index (SFI) using a procedure similar to one used previously.[18] The rats' hind feet were dipped into red ink, and the rats were allowed to walk across a plastic tunnel so that the footprints could be recorded on paper placed at the bottom of the tunnel. The distances between the 3rd and the heel (PL), the 1st and 5th toe (TS), and the 2nd and 4th toe (ITS) were measured on the experimental side (EPL, ETS, and EITS, respectively) and the contralateral normal side (NPL, NTS, and NITS, respectively). The SFI was calculated as[18–20]:

$$\mathrm{SFI} = -38.3 \times (\mathrm{EPL}-\mathrm{NPL})/\mathrm{NPL} + 109.5 \times (\mathrm{ETS}-\mathrm{NTS})/\mathrm{NTS} + 13.3 \times (\mathrm{EITS}-\mathrm{NITS})/\mathrm{NITS} - 8.8$$

In general, the SFI oscillates around zero for normal nerve function; an SFI of −100 represents total dysfunction. An assistant who was blinded to the treatment assignments evaluated the SFI on a weekly basis after surgery. Increased spreading of the toes was considered to be a sign of improved nerve function and gait recovery.

Electrophysiology

The nerve conduction recording of the gastrocnemius muscle was determined with a Nicolet VikingQuest NT system, and data were analyzed and averaged automatically. An incision was made from the gluteus muscles to the popliteal region to expose the sciatic nerve. Supramaximal electric stimulation was applied to the proximal side of the nerve. Compound muscle action potentials (CMAP) were recorded. The distal latency (ms), CMAP amplitude (mV) (peak-to-peak), and area (mm^2) were recorded. The protocol was repeated three times.

Muscle Contraction Force

The gastrocnemius muscle was dissected from the animals, and the weight of the muscle was measured. A wire suture was tied around the distal end of the Achilles tendon, and the suture was attached to a force transducer and amplifier (Statham P23 ID, Oxnard, CA). The sciatic nerve was stimulated directly by an electrical stimulator (SD9 stimulator, GRASS, Testing Laboratories, Inc., Cortland, NY) with increasing amounts of voltage until the maximum isometric single-twitch force was obtained. The force generated was recorded with a calibrated recording oscillograph (RS 3800, Gould) linked to the force transducer. The obtained data were recorded on a computer system (PowerLab/4SP, AD Instruments, Castle Hill, Australia).

Statistics

Student's t-test was used to test for the effects of treadmill training on body weight and SFI. A one-way ANOVA was used for between-group comparisons of gastrocnemius muscle mass, muscle contraction force, CMAP amplitude, and area. Fisher's least significant difference (LSD) test was used for post hoc analyses. The data are presented as the mean ± SD. The analyses were performed using the Statistical Package for the Social Sciences (version 13.0; SPSS, Chicago, IL). Significance was set at $p < 0.05$.

RESULTS

SFI and Walking Track

The rats lost their ability to spread their hind toes after the injection. The between-group analyses of SFI showed that treadmill training improved the functional recovery of gait at 2, 3, and 4 weeks ($p = 0.01$, 0.004, and 0.01, respectively) compared to the control group (Fig. 1).

Body and Muscle Mass

No difference was found in the weekly weight changes for the rats between the groups (Fig. 2A). The mass of

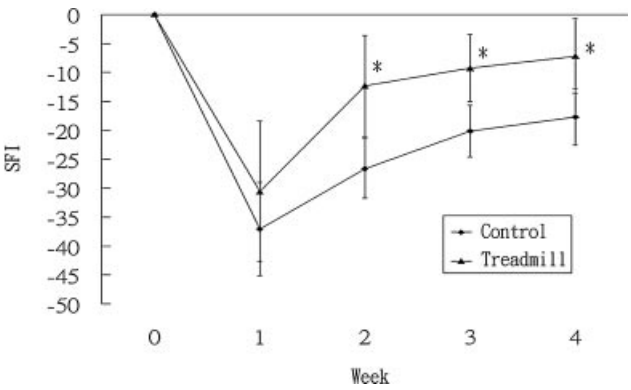

Figure 1. SFI was determined for the control and treadmill groups weekly after BoNT-A injection; $^{*}p < 0.05$.

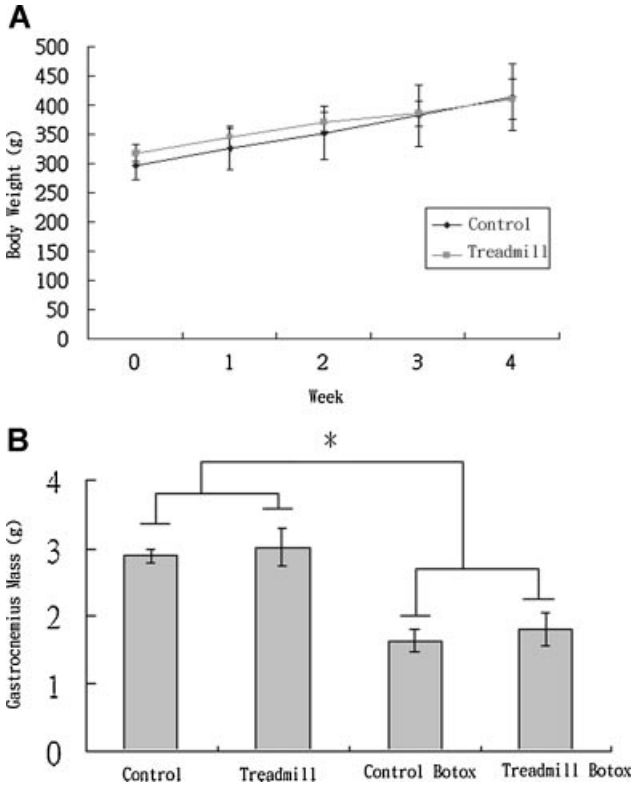

Figure 2. (A) Body mass increased weekly with no difference between the control and treadmill groups. (B) After BoNT-A injection, the gastrocnemius muscles had significantly reduced muscle mass; $^{*}p < 0.05$.

the gastrocnemius muscle removed from the hind limbs was 2.9 ± 0.1 g for the control, 3.1 ± 0.2 g for the treadmill, 1.6 ± 0.2 g for the CB, and 1.8 ± 0.2 g for the TB groups, respectively (Table 1, Fig. 2B). After BoNT-A injection, the mass in the CB and TB groups was significantly reduced ($p < 0.001$) compared to the control and treadmill groups. When the CB and TB groups were compared, no difference was found.

Contraction Force

The maximal contraction force generated in the control and treadmill groups was 40.8 ± 2.7 g and 35.8 ± 5.7 g, respectively ($p = 0.1$). The force generated in the TB group (24.1 ± 6.4 g) was significantly higher ($p < 0.05$) than the force in the CB group (12.3 ± 2.7 g) (Fig. 3).

Electrophysiology

There was no difference in distal latency (ms) between the control (3.3 ± 0.6), treadmill (3.1 ± 0.5), CB (3.4 ± 0.3), and TB (3.6 ± 0.4) groups (Fig. 4A, Table 1). The CMAP amplitude (mV) in the control and treadmill groups was 42.2 ± 11.4 and 47.0 ± 7.0, respectively. The percentage of activated fibers was higher in the TB than in the CB group ($p < 0.05$), which was evident from the increase in the area (TB 14.2 ± 3.3, CB 8.7 ± 2.3) under the curve (Fig. 4B) and in the amplitude (TB 23.2 ± 1.8 mV, CB 17.4 ± 3.5 mV) (Fig. 4C).

DISCUSSION

Our central finding was that after BoNT-A intramuscular injection, treadmill exercise improved the recovery of muscle contraction strength and electrophysiological properties. Muscle paralysis was evident on the 3rd day after receiving BoNT-A injection, and this change was observed throughout the 4-week experimental period. The significantly reduced weights of the gastrocnemius muscles (CB and TB) that were injected with BoNT-A confirmed the paralytic effects of BoNT-A.

After treadmill intervention, the strength on atrophied gastrocnemius (TB) induced by BoNT-A improved significantly. Although the mean gastrocnemius mass in the TB group was higher than in CB, the difference was not significant. This finding was similar to findings of previous studies using running wheel exercise training that found no exercise effect on muscle mass following BoNT-A injection.[21,22] In exercise training, overload is a major mechanism that causes muscle hypertrophy.[23,24] In our treadmill study, because an aerobic mode of exercise was used, the training may not have affected the mass of the gastrocnemius muscle notably. The strength of a muscle depends not only on its size, but also on the properties of the contractile material. Fortuna et al.[25] showed that after BoNT-A injection, the percentage of contractile material was reduced and was replaced primarily by fat. It was previously reported that using treadmill exercise training, the mice had lower percentage of body fat.[26] This fat reduction effect caused by treadmill running may be the other reason why the gastrocnemius mass in the TB group was not significantly

Table 1. Characteristics of Electrophysiology and Gastrocnemius Mass Changes 4 Weeks after BoNT-A Injection and Treadmill Exercise

	Control	CB	Treadmill	TB	p
Latency (ms)	3.3 ± 0.6	3.4 ± 0.3	3.1 ± 0.5	3.6 ± 0.4	0.3
Amplitude (mV)	42.2 ± 11.4	17.4 ± 3.5	47.0 ± 7.0	23.2 ± 1.8	$<0.05^{*}$
Area (mm^2)	27.6 ± 11.4	8.7 ± 2.3	34.4 ± 3.3	14.2 ± 3.3	$<0.05^{*}$
Strength (g)	40.8 ± 2.7	12.3 ± 2.7	35.8 ± 5.7	24.1 ± 6.4	$<0.05^{*}$
Gastrocnemius mass (g)	2.9 ± 0.1	1.6 ± 0.2	3.1 ± 0.2	1.8 ± 0.2	0.9

CB, control botox; TB, treadmill botox. Comparison between CB and TB; $^{*}p < 0.05$.

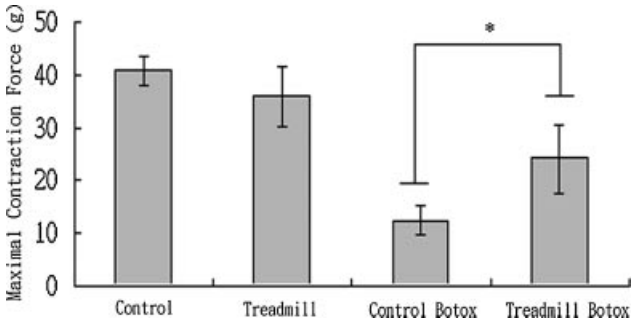

Figure 3. The mean maximal contraction force obtained for the treadmill botox group was significantly higher than the force for the control botox group; $^{*}p < 0.05$.

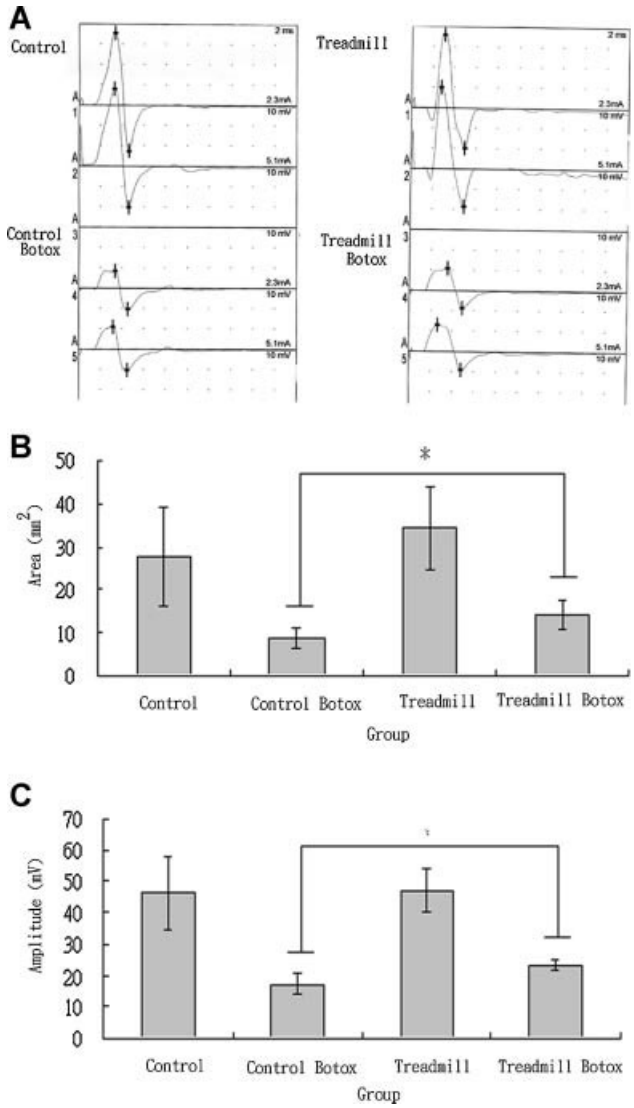

Figure 4. (A) The representative compound muscle action potential waves were elicited in response to electrical stimulation of the sciatic nerve 4 weeks after BoNT-A injection. The time calibration bar is 2 ms, and the amplitude calibration bars are 10 mV. The stimulation period was 0.1 ms. (B) The whole response area under the curve was higher in the treadmill botox (TB) group than in the control botox (CB) group. (C) The mean amplitude of CMAP in the TB group was significantly higher than the amplitude for the CB group; $^{*}p < 0.05$.

higher than in CB. Further investigation may clarify this point.

BoNT-A injections might affect the mass and structural integrity of non-injected contralateral muscles.[25] In one rat model using the contralateral gastrocnemius muscle for comparison, the injected toxin had no effect on the force of the contralateral leg.[27] Dodd et al.[28] concluded that the toxin spreading to contralateral muscles is dependent on dosage. In our study, the contralateral gastrocnemius was used as control. Although the dosage was low (3 units/kg), whether this might have a force-reducing effect on the contralateral leg is unclear. This is a limitation of our study.

In muscle or peripheral nerve injuries, electrophysiological tests are an objective diagnostic tool for evaluating the recovery process. In our study, no change of distal latency was found. This result was comparable to the results of a previous study demonstrating that an injection of BoNT-A causes localized muscle paralysis but no disruption in axonal transport.[29] The CMAP represents the total activity of all muscle action potentials in a muscle or a group of muscles innervated by the same nerve. In chronic inflammatory disease and a nerve transection recovery model, studies showed that CMAP amplitude was correlated with the recovery of muscle strength.[30,31] In our study, a peripheral effect of increased CMAP amplitude was observed after treadmill training. Several underlying mechanisms, including peripheral and central modulations, were proposed to be the mechanisms for the beneficial effects of treadmill training on the recovery process. Decherchi and Dousset[32] showed that central neuron activity adaptation to exercise is activated by exercise-induced changes in muscle metabolism. In another study, alterations in denervated muscle, such as in histochemically stained muscle fiber, and enzyme activities after treadmill training were observed.[33] When lower motor neuron weakness occurred, the CMAP amplitude was determined either by the nerve, the integrity of the neuromuscular junction, or the quality of the muscle innervated by the nerve. Following BoNT-A injection, up-regulation of specific subunits mRNA of nicotinic acetylcholine receptors (nAChR), but not neogenesis of muscle fiber, was reported.[34,35] Whether the treadmill training affects the adaptation of associated gene expression requires additional investigation.

Although the electrophysiological parameters are useful, it is also important to determine the degree of functional recovery. Under experimental conditions, the SFI is typically used for functional assessment of the extent of sciatic nerve injury and for monitoring recovery.[18–20] In sciatic nerve injury, rats lose their ability to spread their hind toes.[36] Our findings suggest that after 4 weeks of treadmill exercise, the improvement in electrophysiological properties and muscle contraction force may lead to improvements in gait.

Our experiments have several implications for clinicians. One of the major effects of local BoNT-A

injection was local muscle weakness and muscle atrophy.[37] In cosmetic applications, the atrophy effect of BoNT-A injection is used for gastrocnemius muscle toning.[38] Whether exercise would counteract this cosmetic atrophy effect remains unknown. Our findings showed that 4 weeks of low-level aerobic exercise did not have hypertrophic effects in gastrocnemius muscle following BoNT-A injection. These findings suggested that subjects receiving BoNT-A gastrocnemius injections for cosmetics purposes can perform lower leg exercises, such as fast walking or jogging, without counteracting the cosmetic effect. Clinically, in spastic patients, such as patients with cerebral palsy or stroke victims, the muscle weakness effect of BoNT-A is typically used to reduce the severity of spasticity. Additionally, treadmill exercise is used as a gait training program for these patients. Based on our findings, the strengthening effect of treadmill exercise may counteract the spasticity reduction effect from BoNT-A. One drawback of our study was that normal rats were used. Currently, no appropriate animal model can mimic the spastic changes of cerebral palsy or stroke in muscle properties.[39] In humans, a stroke may cause spasticity. In a rat stroke model, such as middle cerebral artery ligation or the suture method, paralysis instead of spasticity is typically observed over the contralateral side of the lesion. Commonly used spastic animal models include spinal cord transection, or S2 transection spastic rat tail models that are generated for observation of neuronal overactivity.[39] Thus, it is unclear whether the results of our study can be applied to real spastic muscles. In clinical practice, when considering the therapeutic strategies of combining these two treatments, clinicians should consider this potential counteraction effect. Determining how to avoid the counteracting effect and preserve the neuronal adaptation effect of treadmill training in spastic subjects is an important issue in functional gait training.

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