Increased efficacy and decreased systemic-effects of botulinum toxin A injection after active or passive muscle manipulation

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The effect of physical manipulation on the outcome of neurotoxin (**NT**) injection was studied in a rat tibialis anterior (**TA**) model system where dorsiflexion torque could be measured precisely. After determination of initial torque, all rats received a one-time botulinum toxin A (**BTX-A**) injection (dose 6.0 units/kg in a volume of 100μL) into the **TA** midbelly. Four experimental groups were studied: one group was subjected to BTX-A injection alone (**BTX-A only, *n*=8**), one was subjected to BTX-A injection followed immediately by 10 isometric contractions (**ISO; *n*=9**), and the third was subjected to BTX-A followed immediately by 10 muscle passive stretch/release cycles (**PS; *n*=10**). After 1 month, maximum dorsiflexion torque of the injected and contralateral legs was determined followed by quantification of TA fiber area. Post-injection torque was significantly reduced by around 80% in all NT-treated extremities 1 month after injection (***p*<0.05**). While all NT-treated extremities demonstrated a significant torque decrease relative to their pre-injection levels, ISO and PS groups demonstrated significantly lower torques compared with the BTX-A only group which received no physical manipulation (***p*<0.05**) indicating greater efficacy. Perhaps even more surprising was that the ISO and PS groups both demonstrated a significantly smaller contralateral effect compared with the BTX-A only group that received no manipulation (***p*<0.05**) indicating a decreased systemic-effect. Muscle fiber size generally correlated with dorsiflexion torque. These data demonstrate that both neuromuscular activity (seen in the ISO group) and muscle movement (seen in the PS group) increased the efficacy of BTX-A and decreased the systemic side effects.

Neurotoxins (NT) that block neuromuscular transmission are used to treat muscular spasticity secondary to cerebral palsy, stroke, and head injury.[1–3] Therapeutic effects of NT treatment on spasticity include improved muscle function, facilitation of the effects of physical therapy, and delayed surgery. However, there are immediate and long-term negative side effects related to NT treatments, including systemic weakness and resistance to the toxin.[4–6]

It is suggested that NT side effects are dose-dependent.[1,7] Efforts have been made to identify factors that might improve the effectiveness of this class of drugs, as increased efficacy may result in lower utilized doses, reduced costs, and potentially, longer-lasting treatment effects.

One attempt to increase the efficacy of NT treatment involves activation of the affected muscle. Hughes and Whaler[8] proposed that NT is most efficient in blocking the neuromuscular junction when muscles are activated. This supports the observation that nerve stimulation accelerates toxin internalization into the nerve terminal.[9] While there are reports that both electrical stimulation[1,10,11] and voluntary exercise[12] enhance the effects of NT injection, none of these studies directly measured treated and systemic muscle function. Rather, outcomes were judged based on subjective rating scales and voluntary function. Observed functional changes may thus be due to voluntary activation changes, neuromuscular junction functional changes, muscle fiber size changes, muscle fiber type changes, or a combination of all these factors.

To determine whether physical manipulation, such as electrical stimulation and passive stretch, can alter the efficacy of NT treatment, the present authors developed a high-resolution functional model of muscle torque generation that enables serial testing of joint torque. By using an animal model, the muscle tissue is accessible at the end of the experiment and functional changes can be directly related to the structural properties of the muscle itself. Thus, the aim of this study was to quantify the effect of two physical modalities – isometric contraction (ISO) and passive stretch (PS) – on the functional and structural properties of injected muscles as well as the contralateral muscles that were not injected. The direct effects of physical treatment could be assessed based on the injected muscle's properties, and assessment of the systemic-effects could be based on the contralateral non-injected muscle's properties.

Method

ANIMAL SUBJECTS

Laboratory animals used in this study were untrained, mature male Sprague-Dawley rats (Harlan, Indianapolis, IN, USA) with a mean weight of 392g (standard error of the mean [SEM] 4.6g; *n*=31). Rats were housed two per cage at 20 to 23°C with a 12h:12h dark–light cycle. All procedures were approved by the University of California and the VA Medical Center committees on the ethical use of animal subjects in research. After terminal experiments, animals were sacrificed with an intracardiac injection of pentobarbitol sodium (0.5mL of 390mg/mL solution).
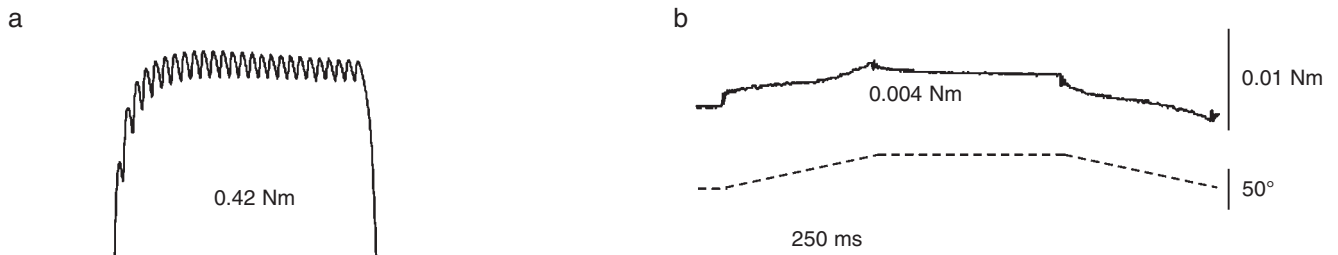
EXPERIMENTAL MODEL

Animal subjects were randomly divided into four groups: one group was subjected to botulinum toxin A (BTX-A, Allergan, Irvine, CA, USA) injection (BTX-A only, *n*=8); the second group was subjected to BTX-A injection followed

immediately by 10 isometric contractions ($n=9$); and the third was subjected to BTX-A followed immediately by 10 muscle passive stretch/release cycles ($n=10$). A fourth group of animals received saline injection to serve as controls for anesthesia, handling, and the injection procedures ($n=4$).

After anesthesia induction (2% isoflurane, 2.0L/min), ankle isometric dorsiflexion torque was measured before injection as previously described.[13] Briefly, dorsiflexors were activated (15V stimulus, 650ms train duration) via the common peroneal nerve while torque was measured using a custom-designed dynamometer. To insure that an intact neuromuscular unit was being tested, the normal neural recruitment pattern

Figure 1: *Mechanical records from experimental manipulation of rat hindlimbs. Solid line represents dorsiflexion torque and dashed line represents ankle joint angle. Both portions of figure are plotted on same time base. (a) Torque record resulting from 40Hz stimulation of peroneal nerve under isometric conditions (note no angular movement). (b) Torque record resulting from passive plantarflexion of ankle of 32°. Note relatively low torque and nonlinear response of dorsiflexion torque to linear angular rotation. Torque level is only about 10% of that observed in panel (a). In this example, peak active torque = 0.41Nm while peak passive torque = 0.004Nm.*
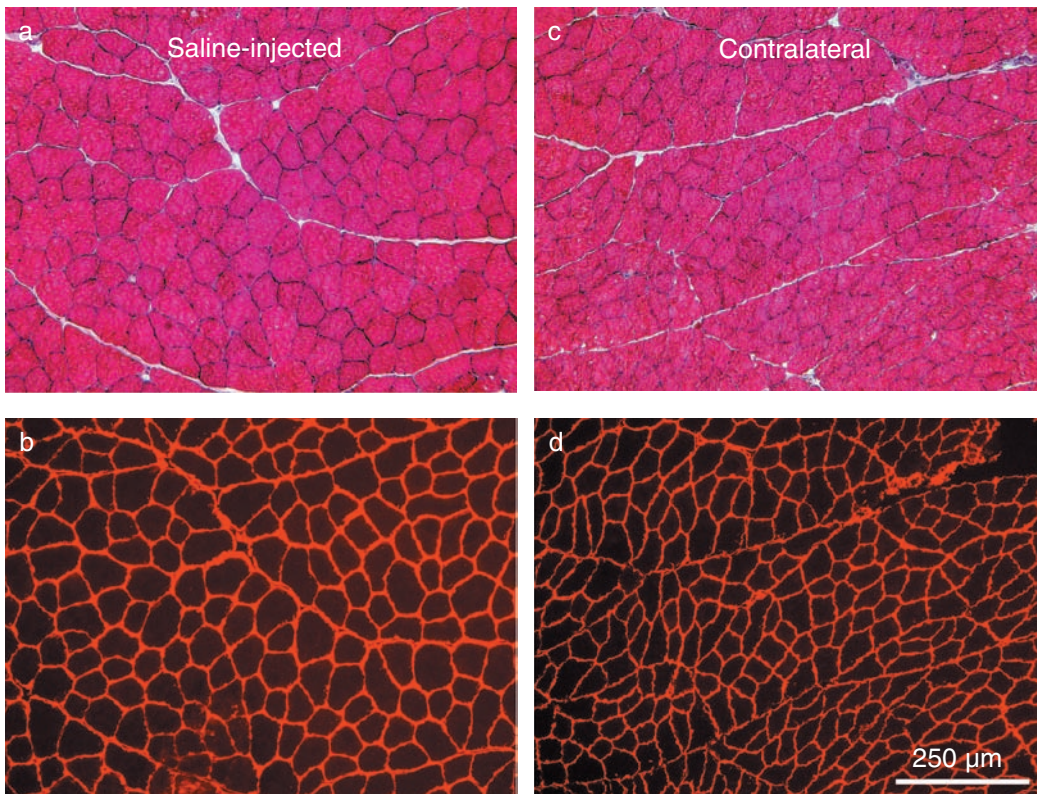
Figure 2: *Serial histological sections from saline-injected muscles. (a, b) saline-injected muscle, (c, d) contralateral muscle. a and c represent haematoxylin and eosin staining for general muscle morphology while b and d represent immunohistochemical sections labeled with a polyclonal antibody to laminin to facilitate computer-assisted quantification of fiber area. Note all sections illustrate normal muscle morphology.*

(increasing torque with increasing stimulation intensity) and force-frequency behavior (increasing torque with increasing stimulation frequency) was observed before injection.

After activating the muscle over the range 20Hz to 100Hz in 20Hz increments, three maximal isometric tetani were elicited at 100Hz. These three contractions were averaged to yield the value for maximal isometric torque which has been shown to have a coefficient of variation of about 10%,[13,14] thus enabling resolution of small changes in dorsiflexor function. After initial torque was determined, all rats received a one-time BTX-A injection (dose 6.0 units/kg in a volume of 100μL) into the midbelly of the tibialis anterior (TA) muscle. This region was localized by palpating the largest bulk of the muscle; the volume was administered by the same physician in two sites of the midbelly. An equal 100μL volume of 0.9% sodium chloride solution was injected in the same way into the control animals' TA muscle.

Application of the physical modalities (ISO or PS) was performed immediately after injection over a 20-minute period. ISO treatment was induced by activating the muscle 10 times at 2-minute intervals and a frequency of 40Hz (15V, train duration of 650ms; Fig. 1a) which results in approximately 65% of maximum tetanic tension that can easily be maintained without fatigue over the 20-minute period (see Fig. 2 of Hentzen et al. 2006).[14]

PS treatment was applied by cyclically plantarflexing the ankle through the tibiotarsal angle range of 90 to 123° over 400ms, also at 2-minute intervals. This results in a peak passive force of <5% maximum isometric tension and linear TA muscle deformation of 12% strain (see Fig. 1b in this article, Fig. 2 of Peters et al. 2003).[13] One month later, dorsiflexion torque was measured from both experimental and contralateral limbs. Animals were then sacrificed and bilateral TA and extensor digitorum longus muscles were excised and weighed.

MUSCLE FIBER SIZE ANALYSIS

Excised TA muscles were snap-frozen in isopentane cooled by liquid nitrogen (–159°C) and stored at –80°C for subsequent analysis. Muscle cross-sections (10μm thick) were taken from TA muscle midbelly. Sections were first treated with 1% bovine serum albumin and normal goat and rat serum as blocking agents. Sections were incubated overnight with a polyclonal anti-laminin antibody (Sigma, St Louis, MO; dilution 1:1000), and then with the secondary antibody, Alexa Fluor 594 goat anti-rabbit immunoglobulin G (Invitrogen, Carlsbad, CA; dilution 1:200). The laminin antibody was used to label the fiber perimeter and facilitate fiber area quantification.

Sections were imaged with a SPOT RT digital camera (Diagnostic Instruments, Sterling Heights, MI) on a Nikon Microphot SA epifluorescent microscope (Nikon, Tokyo, Japan) using a 10× objective with a G-2B filter set for red fluorescence. Based on pilot experiments defining the uniformity of fiber cross-sectional areas in the saline-injected and contralateral muscles from all groups, every third field of view was imaged (Fig. 2). The majority of samples (21 of 23) from the BTX-A injected groups (BTX-A only, ISO, and PS)
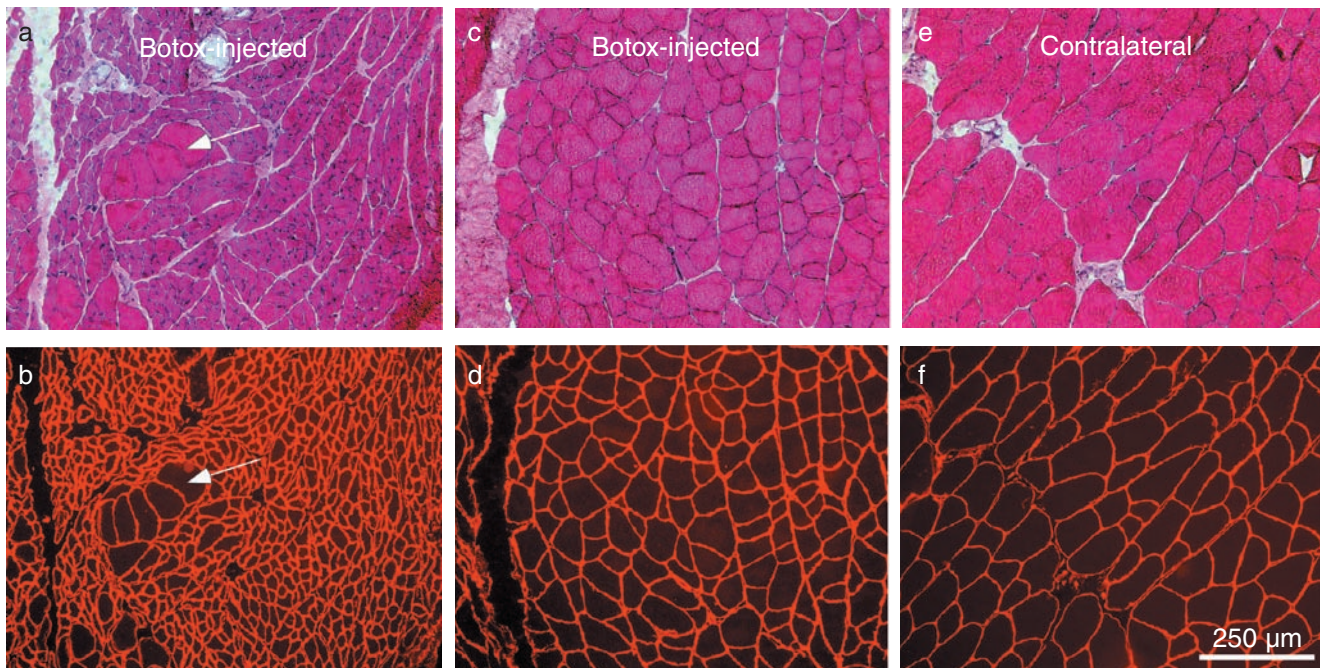
Figure 3: *Serial histological sections of muscles from three treatment groups. (a–d) BTX-A-injected muscle, (e, f) contralateral muscle. Within botulinum toxin A (BTX-A)-injected muscle were severely affected regions (a, b) and less severely affected regions (c, d). These were quantified to determine weighted fiber area. a, c, and e represent haematoxylin and eosin staining for general muscle morphology, while b, d, and f represent immunohistochemical sections labeled with polyclonal antibody to laminin. Note multiple abnormalities in BTX-A-injected muscles including fiber size heterogeneity and increased cellularity. In severely affected region (a, b) some fibers retain normal size and morphology (arrow) even though surrounded by highly atrophic fibers.*

contained a distinct area of severely atrophied fibers which were referred to as the 'severely affected' area surrounded by a less severely affected region. Severely affected areas were easily identified by the highly atrophic and heterogeneous fiber sizes (Figs. 3a, 3b). Images of all fields of view from this severely affected area were recorded. Every other field of view from the less severely affected areas (Figs. 3c, 3d) were also obtained for subsequent quantification.

Based on stereological principles,[13,15] the variance in fiber area was compared with number of fields required for accurate determination in fiber area; the total number of fields quantified from each collection of images was calculated to be five to six. Thus, six images were randomly selected from each group of images, with the use of a random number generator, for cross-sectional area analysis. In cases where there were fewer than six images obtained, all images from that region were analyzed. Of the 21 samples that contained a severely affected area, seven of these samples had fewer than six fields of view in the severely affected region and five samples had fewer than six fields of view in the less severely affected region.

Before analysis, each image was inspected, and areas with sectioning artifacts, blood vessels, merged fibers, or poor staining quality were omitted from the quantification. On average, this protocol resulted in fiber area determination from a mean of 2285 (SEM=210) fibers in injected muscles and 586 (SEM=25) fibers in contralateral muscles, or about 15 and 5% of the entire muscle respectively. To provide a representative average fiber area for the entire section of injected muscles, the fiber area of the severely affected region and the fiber area in the less severely affected region were mathematically averaged, weighted by the area fraction of each region, defined as the area of the region divided by the total area of the muscle cross-section, using the equation:

$$A_s \cdot \Phi_s + A_l \cdot \Phi_l$$

where A_s is the area fraction of the severely affected region, A_l is the area fraction of the less severely affected region, Φ_s is the mean fiber area in the severely affected region, and Φ_l is the mean fiber area of the less severely affected region.

Fiber cross-sectional areas were measured using a custom-written macro in ImageJ (NIH, Bethesda, MD). Filtering criteria were applied to ensure measurement of actual muscle fibers. These criteria rejected regions with areas below $50\mu m^2$ or above $5600\mu m^2$ to eliminate neurovascular structures and 'optically fused' fibers respectively. Fibers touching the edge of the field were excluded as they were assumed to be incomplete. Regions with circularity below 0.30 or above 1.0 were excluded to prevent inclusion of fibers that were obliquely sectioned. Oblique sectioning can artificially increase fiber area through artefact.[15]

STATISTICAL ANALYSIS

Experimental results were analyzed by two-way analysis of variance with repeated measures using treatment group and testing time as grouping factors. Post-hoc Tukey tests were used to compare dependent variables among various pairs of groups. Linear regression was used to determine the association between measured torque and muscle fiber area. All results are reported as mean (SEM) unless otherwise noted.

Results

No significant difference in muscle mass was observed between the injected legs of the three NT-treated groups ($p>0.6$; Fig. 4) or the contralateral legs of the three NT-treated groups ($p>0.7$; Fig. 4). A significant difference was observed between the saline-injected control group and each of the NT-treated groups ($p<0.001$; data not shown). A significant mass decrease was observed for the TA muscle, which was directly injected, as well as the extensor digitorum longus muscles of all NT-treated groups, which share the anterior compartment and the deep peroneal nerve with the TA.

In contrast to the muscle mass result, dorsiflexion torque
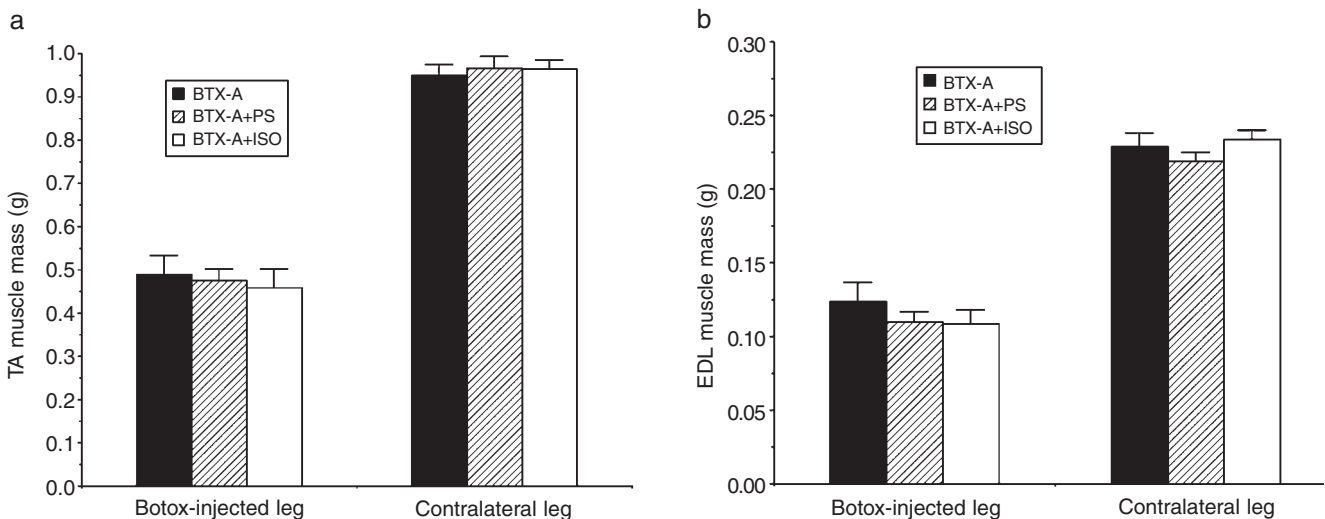
Figure 4: *Muscle mass of botulinum toxin A (BTX-A)-injected and contralateral muscles for each of the experimental groups. (a) Tibialis anterior (TA) muscle mass, (b) extensor digitorum longus (EDL) muscle mass. While a significant difference was observed between legs within animals, no significant difference was observed between experimental treatments (BTX-A only, BTX-A + passive stretch/release cycles [PS], or BTX-A + isometric contractions [ISO]).*

showed a significant effect that was uniquely related to experimental treatment. As expected, post-injection torque was significantly reduced by around 80% in all NT-treated extremities 1 month after injection ($p < 0.05$; Fig. 5) but did not change significantly for saline-injected muscles of controls ($p > 0.7$; data not shown). However, while all NT-treated extremities demonstrated a significant torque decrease relative to their pre-injection levels ($p < 0.05$), the ISO and PS groups (Fig. 5) demonstrated significantly lower torques compared with the BTX-A only group which received no physical manipulation ($p < 0.05$; Fig. 5). Perhaps even more surprising, ISO and PS groups both demonstrated a significantly smaller contralateral effect compared with the BTX-A only group that received no manipulation ($p < 0.05$; Fig. 5).

Muscle fiber size results generally mimicked the functional results presented above. Specifically, fiber size was significantly reduced by about 80% in the severely affected region of all muscles and by about 40% in the less severely affected areas of all NT-treated extremities 1 month after treatment ($p < 0.05$; Fig. 6) relative to the contralateral muscle. The severely affected region of the BTX-A-only injected group was significantly smaller compared with the other two injection groups ($p < 0.01$) and, qualitatively, appeared to be hypercellular (Fig. 3a).

As dorsiflexion torque represents the interaction among peripheral nerve, neuromuscular junction, and muscle functions, researchers wanted to determine whether muscle tissue changes could account for the functional changes described above. To define a fiber area that was functionally relevant, the 'weighted fiber area' calculation described in the Method section was determined.[15] Consistent with the functional results, ISO and PS groups demonstrated significantly larger contralateral fibers compared with the BTX-A-only group ($p < 0.05$; Fig. 7a) indicating a smaller systemic-effect of BTX-A due to physical manipulation even at the cellular level. However, with regard to the injected leg, while the trend was for decreased fiber size in the ISO and PS groups relative to the BTX-A-only group, these results were not statistically significant ($p > 0.14$). Power analysis revealed that a sample size of 19 was required to achieve statistical significance[16] but these experiments were not added to the experimental design.

To determine whether weighted fiber area was functionally relevant, the relationship between fiber area and torque for each animal subject was quantified using linear regression. For all experimental subjects, there was a significant positive relationship between fiber size and joint torque (Fig. 7b, $r^2 = 0.81$, $p < 0.001$). It can be seen that there is a bimodal distribution of the independent variable 'fiber area' which might leverage the regression relationship.[17] To determine whether the regression relationship was compromised by convolution of the four experimental groups, each group was regressed independently, and results from all but the contralateral legs of the saline-injected control muscles were still found to be significant ($p < 0.05$; data not shown).

Discussion

The main result of this study is that the efficacy of BTX-A injection was increased by only 20 minutes of manipulation regardless of whether that manipulation involved active muscle contraction (ISO) or passive stretch. Not only did
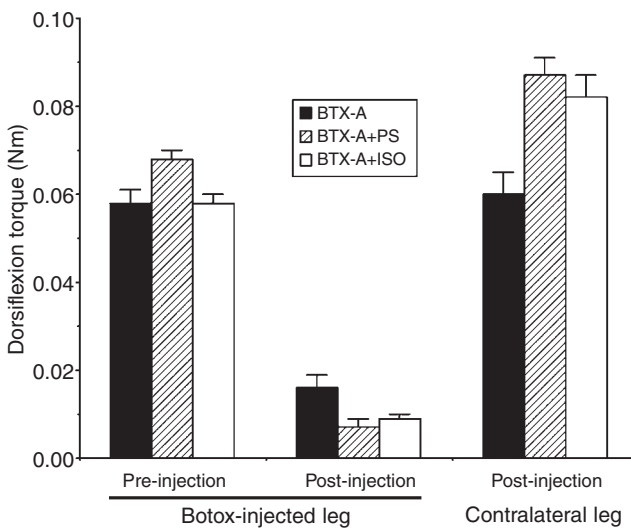
Figure 5: *Dorsiflexion torque measured from botulinum toxin A (BTX-A)-injected leg (two left groups of bars) and the contralateral leg (rightmost bars) for the three experimental treatments (BTX-A only, BTX-A + passive stretch/release cycles [PS], or BTX-A + isometric contractions [ISO]). Note that two groups receiving manipulation (either PS or ISO) showed greater effect in injected leg and a less severe systemic-effect in contralateral leg.*
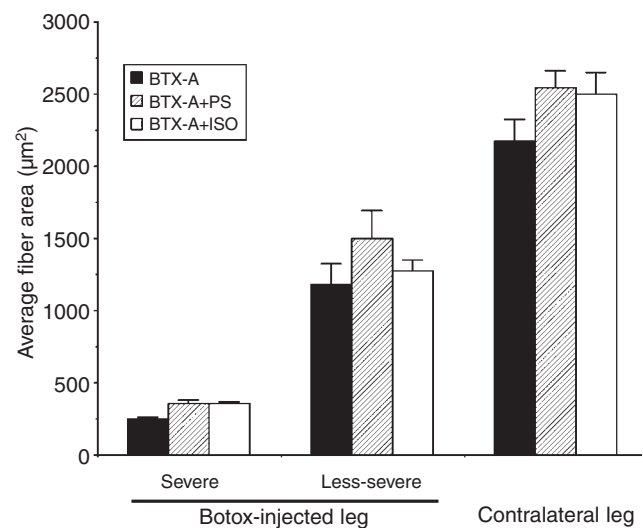
Figure 6: *Muscle fiber area measured in botulinum toxin A (BTX-A)-injected muscles and contralateral muscles for the experimental treatments (BTX-A only, BTX-A + passive stretch/release cycles [PS], or BTX-A + isometric contractions [ISO]). Area is shown for two different regions of the BTX-A-injected muscles: those most severely affected (left bars, also illustrated in Figs. 3a and 3b) and those less severely affected (middle bars, also illustrated in Figs. 3c and 3d respectively).*

manipulation increase the efficacy of BTX-A on the injected muscle, the contralateral muscle was spared the systemic-effects of the toxin with manipulation. This result reveals that muscle motion, regardless of stress or neuromuscular junction activity, increases the efficacy of BTX-A in the manipulated muscle and also spares distant muscles. The mechanism by which this phenomenon occurs is not known.

Previous studies suggested that neural activity would enhance the 'active uptake' of NT into the presynaptic terminal and that any free NT would affect distant muscles, perhaps by diffusion of the toxin.[5,6] Recent basic studies revealed that the SV2 protein is the specific receptor for BTX-A.[18] The receptor binding domain of SV2 is intravesicular, which means that it is only exposed (from the vesicular side of the neurotransmitter vesicle) after fusion with the presynaptic membrane and release of the neurotransmitter acetylcholine. This would be expected to cause preferential binding of the NT for the ISO group. Therefore, it was not expected that PS treatment would promote an increase in NT efficacy. It was anticipated that PS might actually squeeze the 'injectate' out of the muscle, thus decreasing efficacy and increasing the systemic-effect on the contralateral muscles.

A study by Kim et al.[19] demonstrated an enhancement of the paralytic effects of the NT when combining passive stretch and electrical stimulation for 2 hours after BTX-A injection. Although there are several differences between our study and the previous one[19] (Table I), results of both studies emphasize that muscle manipulation may enhance the efficacy of NT injection. Another study demonstrated that NT injection associated with electrical stimulation maximized paralysis, but spontaneous muscle activity appeared earlier in the stimulated muscle compared with the non-stimulated muscle.[10] However, the unique finding of the current study is that the enhancement of NT efficacy is associated with a decreased systemic toxicity of the injection.

Passive stretch in conjunction with NT injection has been studied before using taping,[20,21] which was intended to stretch the spastic muscle progressively for 5 or 6 days. Increased efficacy using this kind of treatment was hypothesized to be due to the production of a muscular activation by elicitation of a tonic stretch reflex.[20] However, this mechanism does not explain the current result, as the PS protocol produced no reflex activation based on the passive torque record which showed the typical nonlinear load-deformation pattern that is observed for most soft tissues (Fig. 1b, solid line). Rather, it appears that any manipulation that 'spreads' the injectate to neuromuscular junctions will have the effect of increased efficacy and decreased systemic-effects. If this is the case, almost any physical manipulation of the muscle could produce the same result, but further studies are necessary to define this mechanism more precisely.

Another interesting aspect of the increased efficacy is the relatively short amount of time required to achieve this result in an animal model: just one 20-minute session. Previous studies carried out in humans report increased BTX-A efficacy but only after much more manipulation time: periodic electrical stimulation over a 24-hour period;[11] 30 minutes of electrical stimulation, six times per day for 3 days after the injection;[1,22] 30 minutes of electrical stimulation per day for 5 days after the injection;[10] and one session of 30 minutes of voluntary exercise.[12]

The current results are also consistent with a previous study demonstrating that increased efficacy of BTX-A when associated with electrical stimulation could result in the use of a decreased dose, as there was no difference in the effectiveness between low dose BTX-A (100 units) combined with short-term stimulation and high-dose (400 units) application in spastic drop foot.[1] The use of a short 20-minute session of manipulation after NT injection is easier and more acceptable to patients compared with the various electrical stimulation
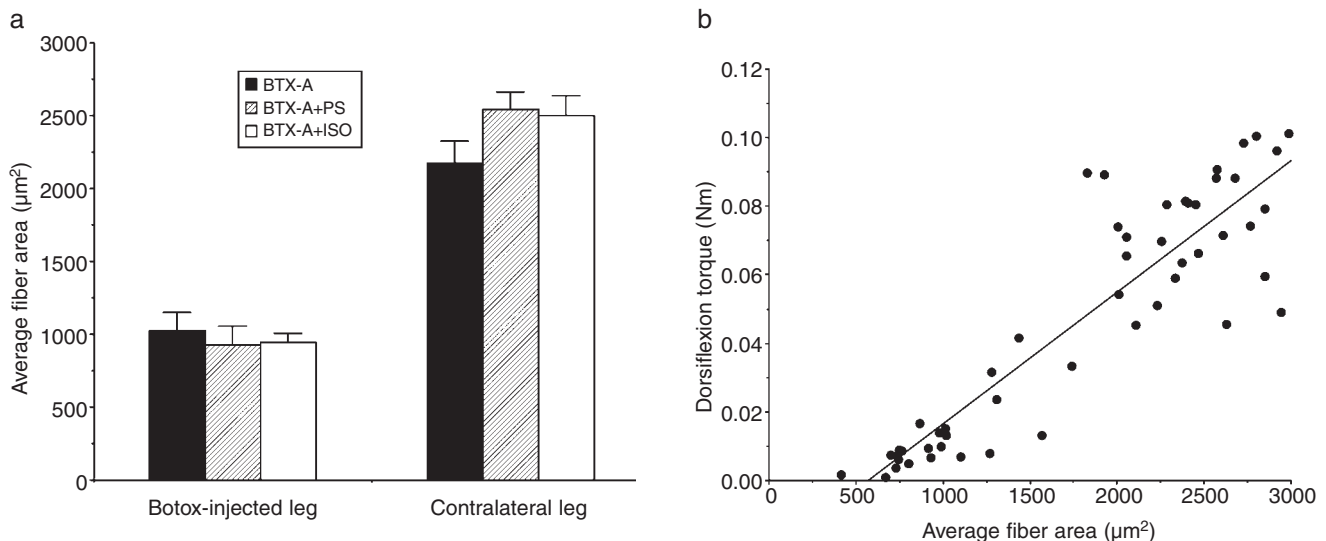
Figure 7: *(a) Overall weighted muscle fiber area measured in botulinum toxin A (BTX-A)-injected muscles and contralateral muscles. Area was calculated using equation in Method section. (b) Correlation between fiber area and dorsiflexion torque for all experimental subjects. Linear regression analysis revealed significant relationship between variables (p<0.001) with coefficient of determination, $r^2=0.81$.*

paradigms. Additionally, passive stretch has an advantage over active muscle contraction and electrical stimulation, both of which are uncomfortable. Passive manipulation does not elicit the pain response which is a commonly-reported symptom after BTX-A injection.

Muscle fiber area quantification clearly showed that the force loss elicited by BTX-A injection was related to muscle fiber force generating capacity, as there was a strong correlation between force generated and fiber size for all muscles studied (Fig. 7b). This is important because neuromuscular junction function or compromised peripheral nerve function could be implicated in the current model which uses indirect muscle fiber activation via the peroneal nerve. It is expected that at this 1-month time point significant nerve sprouting would have occurred and that the elicited function was mediated by sprouted nerves, not the original nerve which would only become functional after about 60 days.[23] Therefore, the sprouted nerves appear to be able to activate the muscle fibers fully under the conditions used here. The fact that fiber size was so dramatically altered by BTX-A injection highlights the extreme sensitivity of muscle fibers to neural activity. The relatively large 80% decrease in fiber size and muscle maximum tetanic tension indicates that the muscle is even more sensitive to 'disuse' by BTX-A injection compared with 'disuse' by other models, such as limb immobilization,[24,25] spaceflight,[26] and hindlimb suspension[27] where 1 month of disuse is usually accompanied by only about a 30% loss in TA function. It is tempting to speculate that loss of neuromuscular junction patency after BTX-A injection may initiate active cellular processes that promote muscle degradation via the ubiquitin-proteasome pathways.[28]

Regarding outcomes of such NT-injection studies, the current data clearly invalidate the use of muscle mass as a measure of function: none of the NT-injection groups showed differences in muscle mass of either the injected or contralateral muscle although both of these groups showed functional differences among them. Thus, if muscle mass had been used in this study as a surrogate for function, this interesting phenomenon would have been completely missed. Muscle mass only provides the crudest of indications of muscle condition, as noncontractile material can contribute to mass (inflammatory cells, fluid, connective tissue, etc.) and muscle tissue mass alone is a poor predictor of function because architectural properties can be altered by disuse treatments.[29,30]

In considering the application of these results to the clinical treatment of spasticity, one must consider several study limitations. First, this animal model used only normal muscle because it is believed that there is no adequate animal model of spasticity.[31,32] As spastic muscles differ from normal muscles both structurally and functionally, further studies are required to determine if similar results to those observed here are seen in spastic muscles. It is possible that the transport of NT is altered by the structural changes that occur within the muscle and it is not even known whether the neuromuscular junctions are normal in spastic muscle. Second, the injection volume used in this study (100μL) was relatively high compared with the volume of the rat TA (~1mL). Based on the mass of the human TA (about 80g),[33] this would be equivalent to injecting 8mL of NT into the muscle which would generally be considered excessive (H Chambers, personal communication 2005). The use of a relatively high injection volume may have overemphasized the magnitude

of the contralateral effect of BTX-A injection. Manipulation did attenuate the contralateral effect in the model system used, and there is no reason to believe that this was specifically due to the relatively large injection volume.

Conclusion

This study demonstrates that either passive or active manipulation of the rat TA muscle after BTX-A injection increased its efficacy in the injected muscle and decreased its systemic side effects to the contralateral muscle. This finding may ultimately be used to define the precise conditions under which the effects of BTX-A injection can be optimized for various human skeletal muscles.

References
1. Bayram S, Sivrioglu K, Karli N, Ozcan O. (2006) Low-dose botulinum toxin with short-term electrical stimulation in poststroke spastic drop foot: a preliminary study. *Am J Phys Med Rehabil* **85:** 75–81.
2. Francisco GE, Boake C, Vaughn A. (2002) Botulinum toxin in upper limb spasticity after acquired brain injury: a randomized trial comparing dilution techniques. *Am J Phys Med Rehabil* **81:** 355–363.
3. Koman LA, Brashear A, Rosenfeld S, Chambers H, Russman B, Rang M, Root L, Ferrari E, Garcia de Yebenes Prous J, Smith BP, et al. (2001) Botulinum toxin type a neuromuscular blockade in the treatment of equinus foot deformity in cerebral palsy: a multicenter, open-label clinical trial. *Pediatrics* **108:** 1062–1071.

Table I: Comparison of current study with findings of Kim et al. (2003)[19]

	Current study	*Kim et al.*
Experimental model		
Animal	Sprague-Dawley rat	New Zealand rabbit
Muscle analyzed	Tibialis anterior	Gastrocnemius
Study design		
BTX-A only	Yes	Yes
Saline injection	Yes	No
BTX-A + PS	Yes	No
BTX-A + ES	Yes	No
BTX-A + PS + ES	No	Yes
PS + ES only	No	Yes
Randomization	Yes	Yes
Contralateral limb control data	Yes	No
Muscle manipulation (PS or ES)		
Duration	20min	2h
Number of sessions	1	1
BTX-A injection		
Dose	6U/kg	6.7 or 8U/kg
Dilution	0.1mL	0.1 or 0.5mL
Evaluation		
Torque	Yes	No
CMAP	No	Yes
Histology time period	4wks	8wks
Measurement intervals		
Baseline	Yes	Yes
1wk	No	Yes
4wks	Yes	Yes
8wks	No	Yes

BTX-A, botulinum toxin A; PS, passive stretch/release cycles; ES, electrical stimulation; CMAP, compound muscle action potential.

4. Chen R, Karp BI, Hallett M. (1998) Botulinum toxin type F for treatment of dystonia: long-term experience. *Neurology* **51:** 1494–1496.
5. Dodd SL, Selsby J, Payne A, Judge A, Dott C. (2005) Botulinum neurotoxin type A causes shifts in myosin heavy chain composition in muscle. *Toxicon* **46:** 196–203.
6. Eleopra R, Tugnoli V, Caniatti L, De Grandis D. (1996) Botulinum toxin treatment in the facial muscles of humans: evidence of an action in untreated near muscles by peripheral local diffusion. *Neurology* **46:** 1158–1160.
7. Mancini F, Sandrini G, Moglia A, Nappi G, Pacchetti C. (2005) A randomised, double-blind, dose-ranging study to evaluate efficacy and safety of three doses of botulinum toxin type A (Botox) for the treatment of spastic foot. *Neurol Sci* **26:** 26–31.
8. Hughes R, Whaler B. (1962) Influence of nerve-ending activity and of drugs on the rate or paralysis of rat diaphragm preparation by Cl. Botulinum type A toxin. *J Physiol (London)* **160:** 221–233.
9. Black JD, Dolly JO. (1986) Interaction of 125I-labeled botulinum neurotoxins with nerve terminals. II. Autoradiographic evidence for its uptake into motor nerves by acceptor-mediated endocytosis. *J Cell Biol* **103:** 535–544.
10. Frasson E, Priori A, Ruzzante B, Didone G, Bertolasi L. (2005) Nerve stimulation boosts botulinum toxin action in spasticity. *Mov Disord* **20:** 624–629.
11. Eleopra R, Tugnoli V, De Grandis D. (1997) The variability in the clinical effect induced by botulinum toxin type A: the role of muscle activity in humans. *Mov Disord* **12:** 89–94.
12. Chen R, Karp BI, Goldstein SR, Bara-Jimenez W, Yaseen Z, Hallett M. (1999) Effect of muscle activity immediately after botulinum toxin injection for writer's cramp. *Mov Disord* **14:** 307–312.
13. Peters D, Barash IA, Burdi M, Yuan PS, Mathew L, Fridén J, Lieber RL. (2003) Asynchronous functional, cellular and transcriptional changes after a bout of eccentric exercise in the rat. *J Physiol (London)* **553:** 947–957.
14. Hentzen E, Lahey M, Peters D, Mathew L, Barash I, Fridén J, Lieber R. (2006) Stress-dependent and stress-independent expression of the myogenic regulatory factors and the MARP genes after eccentric contractions. *J Physiol (London)* **570:** 157–167.
15. Weibel ER. (1979) *Stereological Methods. Vol 1 – Practical Methods for Biological Morphometry.* London: Academic Press. pp 9–62, 101–161.
16. Sokal RR, Rohlf FJ. (1981) *Biometry.* San Francisco: WH Freeman and Company.
17. Draper NR, Smith H. (1981) *Applied Regression Analysis.* New York: John Wiley & Sons.
18. Dong M, Yeh F, Tepp WH, Dean C, Johnson EA, Janz R, Chapman ER. (2006) SV2 is the protein receptor for botulinum neurotoxin A. *Science* **312:** 592–596.
19. Kim HS, Hwang JH, Jeong ST, Lee PKW, Shim JSS. (2003) Effect of muscle activity and botulinum toxin dilution volume on muscle paralysis. *Dev Med Child Neurol* **45:** 200–206.
20. Carda S, Molteni F. (2005) Taping versus electrical stimulation after botulinum toxin type A injection for wrist and finger spasticity. A case–control study. *Clin Rehabil* **19:** 621–626.
21. Molteni F. (1995) Botulinum toxin and rehabilitation programs in lower limb spasticity. *Eur J Neurol* **2:** 61–67.
22. Hesse S, Jahnke MT, Luecke D, Mauritz KH. (1995) Short-term electrical stimulation enhances the effectiveness of Botulinum toxin in the treatment of lower limb spasticity in hemiparetic patients. *Neurosci Lett* **201:** 37–40.
23. De Paiva A, Meunier F, Molgó J, Aoki K, Dolly O. (1999) Functional repair of motor endplates after botulinum neurotoxin type A poisoning: biphasic switch of synaptic activity between nerve sprouts and their parent terminals. *Proc Natl Acad Sci USA* **96:** 3200–3205.
24. Booth FW. (1982) Effect of limb immobilization on skeletal muscle. *J Appl Physiol* **52:** 1113–1118.
25. Pattullo M, Cotter M, Cameron N, Barry J. (1992) Effects of lengthened immobilization on functional and histochemical properties of rabbit tibialis anterior muscle. *Exp Physiol* **77:** 433–442.
26. Caiozzo V, Haddad F, Baker M, Herrick R, Prietto N, Baldwin K. (1996) Microgravity-induced transformations of myosin isoforms and contractile properties of skeletal muscle. *J Appl Physiol* **81:** 123–132.
27. Roy R, Bello M, Bouissou P, Edgerton R. (1987) Size and metabolic properties of fibers in rat fast-twitch muscles after hindlimb suspension. *J Appl Physiol* **62:** 2348–2357.
28. Mitch WE, Goldberg AL. (1996) Mechanisms of muscle wasting. The role of the ubiquitin-proteasome pathway. *N Engl J Med* **335:** 1897–1905.
29. Simard CP, Spector SA, Edgerton VR. (1982) Contractile properties of rat hindlimb muscles immobilized at different lengths. *Exp Neurol* **77:** 467–482.
30. Spector SA, Gardiner PF, Zernicke RF, Roy RR, Edgerton VR. (1980) Muscle architecture and force-velocity characteristics of the cat soleus and medial gastrocnemius: implications for motor control. *J Neurophysiol* **44:** 951–960.
31. Lieber RL, Steinman S, Barash IA, Chambers H. (2004) Structural and functional changes in spastic skeletal muscle. *Muscle Nerve* **29:** 615–627.
32. Wright J, Rang M. (1990) The spastic mouse. And the search for an animal model of spasticity in human beings. *Clin Orthop Relat Res* **253:** 12–19.
33. Wickiewicz TL, Roy RR, Powell PL, Edgerton VR. (1983) Muscle architecture of the human lower limb. *Clin Orthop Rel Res* **179:** 275–283.

List of abbreviations

BTX-A	Botulinum toxin A
ISO	Isometric contractions
NT	Neurotoxin
PS	Passive stretch/release cycles
TA	Tibialis anterior