

DRAMATIC CHANGES IN MUSCLE CONTRACTILE AND STRUCTURAL PROPERTIES AFTER 2 BOTULINUM TOXIN INJECTIONS

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Accepted 8 January 2015

ABSTRACT: *Introduction:* Botulinum toxin is frequently administered serially to maintain therapeutic muscle paralysis, but the effect of repeated doses on muscle function are largely unknown. This study characterized the muscle response to 2 onabotulinum toxin (BoNT) injections separated by 3 months. *Methods:* Animal subjects received a single toxin injection ($n = 8$), 2 BoNT injections separated by 3 months ($n = 14$), or 1 BoNT and 1 saline injection separated by 3 months ($n = 8$). *Results:* The functional effect of 2 serial injections was exponentially greater than the effect of a single injection. While both groups treated with a single BoNT injection had decreased torque in the injected leg by approximately 50% relative to contralateral legs, the double BoNT injected group had decreased torque by over 95% relative to the preinjection level. Both single and double BoNT injections produced clear signs of fiber-type grouping. *Conclusions:* These experiments demonstrate a disproportionately greater effect of repeated BoNT injections.

Muscle Nerve 52: 649–657, 2015

Muscle spasticity can result from upper motor neuron lesions, potentially leading to movement impairment and muscle contractures. One of the most widely used pharmacological interventions for spasticity is onabotulinum toxin A [(Botox[®]); BoNT]. This neurotoxin paralyzes injected muscle by cleaving one of the SNARE complex proteins required for synaptic vesicle exocytosis,^{1,2} inhibiting acetylcholine release at the neuromuscular junction (NMJ). Because the drug's effect on nerve terminals is reversible,³ repeat BoNT injections are necessary to maintain the functional benefit of muscle paralysis.

Despite reports of adequate muscle function after repeated BoNT injections in cerebral palsy treatment⁴ and other neurological disorders,^{5,6} two previous studies observed intramuscular lipid accumulation after multiple BoNT injections,

indicating a pathological muscle response.^{7,8} As the BoNT doses used were higher and administered more frequently than usual, it is not clear if these results have direct clinical application. Another indicator of normal muscle structure is the typical mosaic pattern of fiber types within the muscles, which indicates a normal innervation pattern. This was not evaluated in either study, so the effect of repeated injections on reinnervation was not explored.

We previously reported the effects of BoNT in a high-resolution functional muscle assay in which the effects of joint manipulation⁹ and injectate dose and volume¹⁰ were investigated. These studies have allowed us to define the “normal” structural and functional response to a single BoNT injection. Given previous reports of pathological response to multiple injections and the general clinical impression that BoNT injection efficacy decreases with time,¹¹ we were interested in determining whether repeat injections cause the same effect as the initial injection (i.e., same relative decline in muscle function) or whether a second injection resulted in a decreased or increased atrophic response by the muscle. We hypothesized that multiple muscle injections would cause a disproportionately smaller atrophic response compared with a single injection, because this is the general clinical impression. Thus, the purpose of this study was to quantify muscle functional and structural properties in the adult rat tibialis anterior muscle (TA) in a clinically relevant scenario following 2 injections of BoNT separated by 3 months. For the purposes of this study, “function” refers strictly to the maximum tetanic tension of the skeletal muscle measured as described below in contrast to true patient function reflected by functional activities of daily living.

MATERIAL AND METHODS

Animal Subjects. Laboratory animals used in this study were untrained, mature male Sprague-Dawley rats (Harlan, Indianapolis, Indiana) with an average size of 422.0 ± 7.6 g (mean \pm standard error of the mean, SEM, $n = 30$). Rats were housed 2 per cage at 20–23 °C with a 12 h:12 h dark–light cycle.

Abbreviations: ANOVA, analysis of variance; BoNT, onabotulinum toxin A (Botox[®]); BSA, bovine serum albumin; NMJ, neuromuscular junction; TA, tibialis anterior muscle

Key words: Botox[®]; denervation; fiber type; muscle physiology; neurotoxin

Funding for this study was provided by the Department of Veterans Affairs (grant RX000670), NIH (grants R24HD050837 and AR057013), and Allergan, Inc.

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Published online 19 January 2015 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/mus.24576

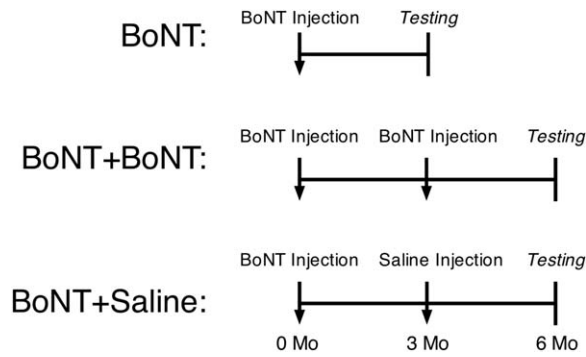


FIGURE 1. Schematic diagram of experimental procedures. Animal subjects were divided randomly into 3 groups: a single toxin injection ($n = 8$), 2 botulinum toxin injections separated by 3 months ($n = 14$), or 1 toxin and 1 saline injection separated by 3 months ($n = 8$). The latter group was used to insure that a time and age-matched comparison could be made to the BoNT+BoNT group.

The Institutional Animal Care and Use Committee of the Veterans Affairs San Diego Healthcare System approved the experimental methods. After terminal experiments, animals were killed by intracardiac injection of pentobarbital sodium (0.5 ml of 390 mg/ml solution).

Experimental Model. Animal subjects were randomly divided into 3 groups (Fig. 1): a single toxin injection (BoNT; $n = 8$), 2 BoNT injections separated by 3 months (BoNT+BoNT; $n = 14$), or 1 BoNT and 1 saline injection separated by 3 months (BoNT+Saline; $n = 8$). The latter group was used to insure that a time and age-matched comparison could be made to the BoNT+BoNT group. Contralateral, uninjected hindlimbs were also evaluated. After anesthesia induction (2% isoflurane, 2.0 L/min), rats received a single 100- μ l injection of BoNT (6.0 units/kg) into the midbelly of the right TA muscle. The muscle midbelly was localized by palpating the largest muscle bulk, and the volume was administered as described previously in detail.⁹ Three months later, animals received 1 of the following: measurement of isometric torque as reported previously,¹² a second 100- μ l injection of BoNT, or a 100- μ l injection of normal saline (Fig. 1). Three months later, isometric torque was measured in the 2 injection groups. Because all animals began the experiment at the same age, the double injection groups were 3 months older than the single injection group at the time of torque measurement.

To measure torque, dorsiflexors were activated (15 V stimulus, 650 ms train duration) by stimulation of the common fibular nerve while torque was measured using a custom-designed dynamometer. After activating the muscle over the range 20 Hz to 100 Hz in 20 Hz increments, 3 maximal isometric tetani were elicited at 100 Hz. These 3

contractions were averaged to yield the value for maximal isometric torque, which has a coefficient of variation of approximately 10%,^{12,13} enabling resolution of small changes in dorsiflexor function. Animals were then killed, and bilateral TA muscles were excised and weighed.

Muscle Fiber Analysis. Excised TA muscles were snap-frozen in isopentane cooled by liquid nitrogen (-159°C) and stored at -80°C for subsequent analysis as described previously.⁹ Briefly, muscle cross-sections (10 μm thick) were taken from the TA muscle midbelly and treated with 1% bovine serum albumin (BSA) and normal goat and rat serum as blocking agents. Sections were incubated overnight with a polyclonal anti-laminin antibody (Sigma, St Louis, Missouri; dilution 1:1,000), and then with the secondary antibody, Alexa Fluor 594 goat anti-rabbit IgG (Invitrogen, Carlsbad, California; dilution 1:200). The laminin antibody is used to label the fiber perimeter and facilitate fiber area quantification.

Sections were imaged with a SPOT RT digital camera (Diagnostic Instruments, Sterling Heights, Michigan) on a Nikon Microphot SA epifluorescent microscope (Nikon, Tokyo, Japan) using a 10 \times objective with a G-2B filter set for red fluorescence. Based on previous sampling studies,⁹ every other field of view from injected muscles was imaged, and 12 images per section were quantified. In contralateral muscles, every third field of view was imaged, and 6 images per section were quantified. Before analysis, each image was inspected, and areas with sectioning artifacts, blood vessels, merged fibers, or poor staining quality were omitted. Muscle fiber cross-sectional area was measured using a custom-written macro in ImageJ (NIH, Bethesda, Maryland).

For quantification of fiber type grouping, we exploited the fact that there are less than 1% type 1 (slow) fibers in the rat TA muscle (see Table 2 of Eng et al. 2010). This number, which ranges from 200 to 400 fibers in a normal TA muscle, ensures that almost every type 1 fiber exists as a single cell surrounded by type 2 (fast) fibers. Thus, if fiber-type grouping occurs due to reinnervation of a cluster of fibers, it can be detected morphologically without sophisticated single motor unit experiments. Frozen sections were first treated with 1% BSA and normal horse and rat serum as blocking agents and then incubated overnight with a monoclonal anti-slow myosin heavy chain antibody (WB-MHCs, Vector Laboratories, Burlingame, California; dilution 1:500). Antibody reactivity was visualized using the indirect immunoperoxidase technique (Vectastain Elite ABC Kit, Vector Laboratories). Fiber type grouping was then quantified

by scanning the entire section with a 10× objective through a Leica DFC295 digital camera on a Leica DM6000 B microscope (Leica, Wetzlar, Germany). A “tile scan” was created by stitching together all individual images (ranging from 12–104) using the Leica LAS AF software to create a single large image representing the entire muscle section. Every myosin heavy chain 1 (MyHC1) positive fiber was counted as part of a “group” defined as the total number of fibers touched by another MyHC1 positive fiber. This number ranged from 1 (no grouping) to 24 (severe grouping). For each experimental group, the percentage of fibers in groups of 1, 2, 3, 4, etc., was quantified to yield a fiber-type grouping histogram for that muscle. These values were then binned and averaged across experimental animal subjects.

Intramuscular Lipid Accumulation. Intramuscular lipid accumulation was assessed using Oil Red O staining (American MasterTech, Lodi, California) of cross-sections obtained from the muscle mid-belly. Sections were imaged with the SPOT RT digital camera on a Nikon Microphot SA epifluorescent microscope using a 1× objective.

Myosin Heavy Chain (MyHC) Analysis. MyHC isoform distribution was determined using methods described.¹⁴ Briefly, a small piece from the muscle mid-belly was homogenized, and the myofibril-rich pellets were washed and resuspended in buffers supplemented with protease inhibitor cocktail (5 μ l of 100 mM phenylmethanesulfonyl fluoride, 10 μ g/ μ l leupeptin, and 10 μ g/ μ l pepstatin A). Protein was then diluted in sample buffer to a concentration of 0.125 mg/ml. Separation of MyHC isoforms was performed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (16.5 cm by 22 cm, thickness: 0.75 mm) with 22 h of migration at 275 V at 4 °C. Stacking and resolving gels were 4% and 8% polyacrylamide, respectively. After migration, gels were silver stained according to the manufacturer’s instructions (Bio-Rad, Hercules, California), and relative isoform content was determined by scanning the gels on a calibrated densitometer (GS-800, Bio-Rad) and performing analysis using Quantity One (Bio-Rad). The positions of MyHC isoforms were determined by their relative electrophoretic mobilities, which have been characterized previously.¹⁴

Collagen Content. Traditional methods of expressing “fibrosis” in terms of cross-section area fraction of connective tissue are subject to ambiguity when significant muscle fiber atrophy occurs, as in this model. Thus, to assay for the effect of BoNT on connective tissue, we estimated the amount of extracellular matrix material based on total

collagen content. The value was calculated by measuring hydroxyproline content. Briefly, portions of the muscle that contained no internal tendon were isolated and hydrolyzed in 6N HCl at 110 °C for 18 h. After hydrolysis, samples were neutralized and treated with a chloramine T solution for 20 min at room temperature followed by a solution of p-diaminobenzaldehyde for 30 min at 60 °C. Sample absorbance was read from each sample in triplicate at 550 nm and compared with the standard curve to quantify hydroxyproline content. Hydroxyproline content was then converted to collagen content using the constant (7.46) defining the number of hydroxyproline residues in a molecule of collagen.

Statistical Analysis. Experimental results were analyzed by 2-way analysis of variance using treatment group and side as grouping factors. *Post hoc* Bonferroni tests were used to perform multiple paired comparisons between pairs of groups. All results are reported as mean \pm SEM unless otherwise noted.

RESULTS

The functional muscle effect of serial BoNT injections was quite different from the effect of a single injection on both the absolute and relative scales. This was indicated statistically by the 2-way analysis of variance (ANOVA) of dorsiflexion torque, which revealed a significant effect of side (injected vs. contralateral, $P < 0.001$), a significant effect of treatment (among the 3 experimental groups; $P < 0.001$), and a significant side \times treatment interaction ($P < 0.001$; Fig. 2A). There was no significant difference between injected legs given either a single BoNT dose or a single BoNT dose followed by a single saline dose ($P > 0.6$; compare open and hatched bars in Fig. 2A), indicating that the 3 month age difference did not affect dorsiflexion torque. However, the experimental leg treated with 2 serial BoNT doses was significantly weaker compared with the other treatments ($P < 0.001$; Fig. 2A). Specifically, while both legs injected with a single BoNT injection only generated approximately 50% of the dorsiflexion torque of the contralateral leg after 3 months, the double BoNT injected leg remained essentially completely paralyzed (torque decreased by over 95% relative to BoNT or BoNT+Saline groups). Thus, the second BoNT injection was more effective (greater decrement in muscle function and more persistent) compared with the first injection. This result was roughly but not perfectly paralleled by changes measured in skeletal muscle mass (Table 1).

Structural parameters qualitatively paralleled functional results. For example, muscle fiber cross-sectional area, a surrogate for the total amount of

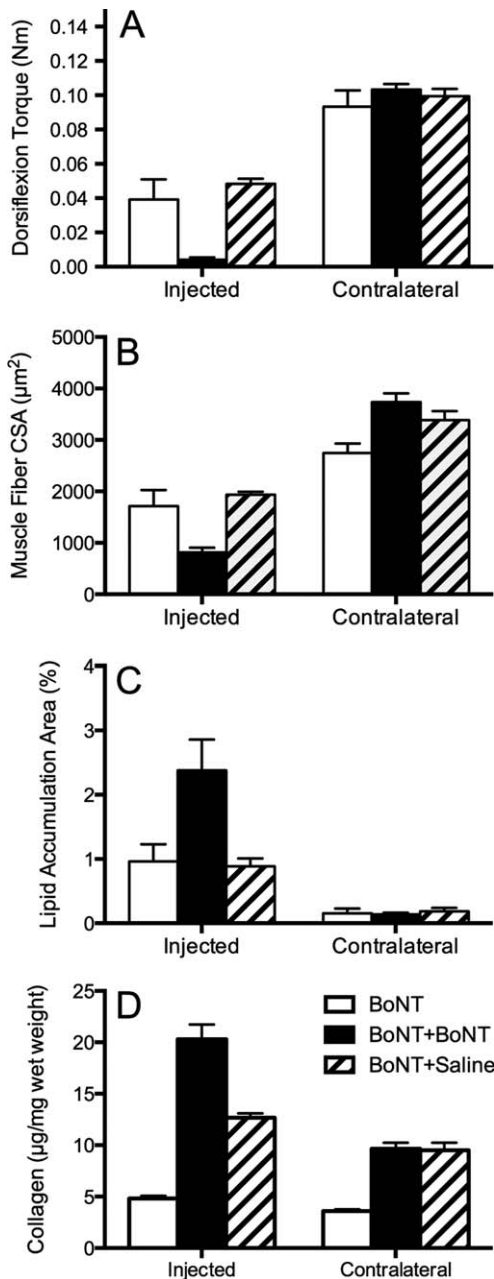


FIGURE 2. (A) Torque measured from injected and contralateral hindlimbs. Note a reduction in torque in all BoNT injected limbs compared with contralateral uninjected muscle ($P < 0.00001$). In the injected limbs, torque was reduced significantly in the double BoNT injected group compared with other groups ($P < 0.0001$). (B) Average muscle fiber CSA measured in injected BoNT hindlimb and contralateral uninjected side. There was a significant decrease in fiber CSA in double BoNT injected muscle compared with single injected muscle, in BoNT followed by saline injected muscle ($P < 0.0001$), and in all injected muscles when compared with contralateral uninjected muscles ($P \leq 0.05$). (C) Percentage of lipid accumulation area in injected and contralateral uninjected TA muscles. Note a higher percentage of lipid accumulation in double BoNT injected muscles compared with contralateral ($P < 0.0001$). (D) Collagen content of the experimental groups measured by hydroxyproline content. Collagen content increased in both double injection groups. This appears to be more of an effect of age than neurotoxin injection, because the second saline injection elicited the same response.

Table 1. Injected and contralateral tibialis anterior muscle masses (g)*

Group	Injected	Contralateral
BoNT	0.74 ± 0.04 [†]	1.05 ± 0.02 [‡]
BoNT + BoNT	0.31 ± 0.01 [§]	1.05 ± 0.01 [‡]
BoNT + Saline	0.62 ± 0.02	0.99 ± 0.03 [‡]

*N = 8-14/group (see Materials and Methods section). Values represent mean ± SEM.

[†]When compared to BoNT + Saline group.

[‡]When compared to injected muscle ($P < 0.0001$).

[§]When compared to BoNT and BoNT + Saline groups ($P < 0.0001$).

contractile material present, showed the same 2-way ANOVA results (significant effect of side, $P < 0.001$; significant effect of treatment, $P < 0.05$; significant side × treatment interaction, $P < 0.001$; Fig. 2B). While muscle fiber size decreased by approximately 35% for the singly injected experimental legs relative to contralateral legs, the double BoNT injection decreased fiber size by over 75%. In other words, the second dose had a greater effect compared with the first.

A very small amount of lipid was measured in the injected muscles of all groups (~1–3% relative to total muscle cross-sectional area). Thus, this particular injection paradigm did not elicit a dramatic lipogenic response, but the amount of lipid was, nevertheless, abnormal. Noninjected muscles show virtually no lipid (<0.2%). Two-way ANOVA revealed a significant effect of side ($P < 0.001$), a significant effect of treatment ($P < 0.05$), and a significant side × treatment interaction ($P < 0.05$; Fig. 2C). While both single injection experimental muscles showed approximately a 7-fold increased lipid accumulation, the double injected muscle showed over a 12-fold increase. Morphological appearance of lipid was relatively superficial in the muscle belly and appeared to collect in clusters in the perimysial space, but not necessarily in areas containing the most significantly atrophied fibers (arrows, Fig. 3).

Myosin heavy chain composition reflects the overall level of muscle use. Muscles that experience increased levels of use tend to increase their type 1 fiber type percentage, and muscles with decreased level of use have increased type 2 fiber type percentage.¹⁵ Fiber subtypes can also shift within the fast fiber type from 2A to 2X to 2B (slowest to fastest) as muscle use decreases. The myosin heavy chain profiles of all injected muscles shifted slightly but significantly toward the slower phenotype with a significant decrease in type 2B and significant increase in type 1 and type 2A fiber type percentage (Table 2). The double injected leg increased in this direction to a significantly greater extent compared with either singly injected leg

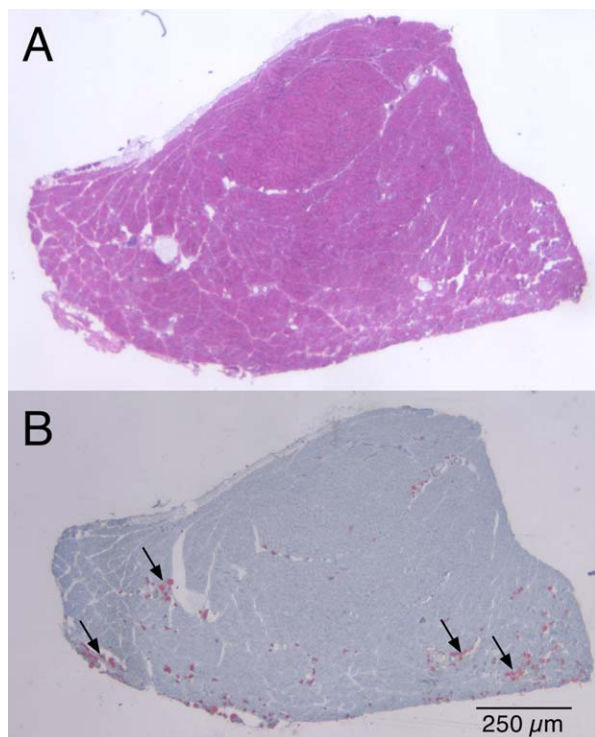


FIGURE 3. TA cross-section from a BoNT injected muscle. **(A)** Traditional hematoxylin and eosin reflects general morphology. **(B)** Oil Red O staining reveals red droplets that represent fat accumulation, which was not observed in any control muscles.

($P < 0.05$). Neither a side nor treatment effect was observed for type 2X fibers, but this was expected because, in the process of transformation from type 2B to type 1, only the extreme fiber types typically demonstrate significant changes.¹⁶

Both single and double BoNT injection produced signs of fiber type grouping. In control skeletal muscles, $85 \pm 1\%$ of type 1 fibers were completely surrounded by type 2 fibers (Fig. 4, open bar, number of fibers in “group” equals 1) while in all 3 of the BoNT groups, less than 70% of type 1 fibers were completely surrounded by type 2 fibers ($P < 0.001$; Fig. 4, black, hatched, and grey bars). Fiber type grouping was obvious in all of the BoNT injected groups (note significant differences between control fibers and all other

groups with fiber clusters of 2–6) with maximum numbers of fibers in a cluster ranging from 16 to 24, while control tissue had a maximum of 6 fibers in a group, as was evident visually on the micrographs (Fig. 5).

Collagen Content. Collagen content in the BoNT double-injected group was higher compared with either the single BoNT injection group or contralateral muscles ($P < 0.0001$). This was demonstrated statistically by the 2-way ANOVA of collagen content, which revealed a significant effect of side (injection vs. contralateral, $P < 0.0001$), a significant effect of treatment (among the 3 experimental groups; $P < 0.0001$), and a significant side x treatment interaction ($P < 0.0001$; Fig. 2D).

DISCUSSION

The purpose of this study was to determine the effect of a second BoNT injection into a muscle that had been injected previously. We hypothesized that the second dose would have a relatively smaller functional effect based on the literature and clinical experience, but this hypothesis was soundly rejected based on our functional and structural measurements.

It is interesting that numerous studies have shown both safety and efficacy of repeated BoNT injections to reduce spasticity, improve muscle tone, and functional mobility.⁶ In these studies, patients received 3 to 5 injections of BoNT, and a minimum of 3 months elapsed between treatment cycles. Subsequent injections were performed when deemed clinically necessary. Our results suggest that a subsequent injection actually has a much greater relative effect on the muscle compared with the initial injection, at least in normal muscle. In functional terms, we found that, while the initial injection decreased torque by approximately 50% 3 months postinjection, the subsequent injection almost completely eliminated muscle function, decreasing torque by approximately 95% (Fig. 2A). This appears to be due to the fact that the muscle had not recovered by the 3-month period, even though this is the period

Table 2. Myosin heavy chain distribution (%)*

Group	MyHC1		MyHC2A		MyHC2X		MyHC2B	
	Injected	Contralateral	Injected	Contralateral	Injected	Contralateral	Injected	Contralateral
BoNT	4.8±0.5	1.7±0.3 [†]	15.3±2.0	7.6±0.9 [†]	50.3±2.0	43.0±3.9	29.8±3.0	47.5±4.6 [†]
BoNT + BoNT	8.6±0.8 [‡]	1.5±0.2 [†]	28.7±1.7 [‡]	4.8±1.5 [†]	44.3±1.2	46.0±2.8	18.3±1.9	47.5±2.4 [†]
BoNT + Saline	3.8±0.6	1.0±0.1 [†]	15.3±1.2	7.5±0.6 [†]	54.1±2.6	44.5±4.0	26.7±2.6	47.0±4.0 [†]

*N = 8-14/group (see Materials and Methods section). Values represent mean ± SEM.

[†]When compared to injected muscle ($P < 0.05$).

[‡]When compared to BoNT and BoNT + Saline ($P < 0.0001$).

MyHCx, myosin heavy chain type “X.”

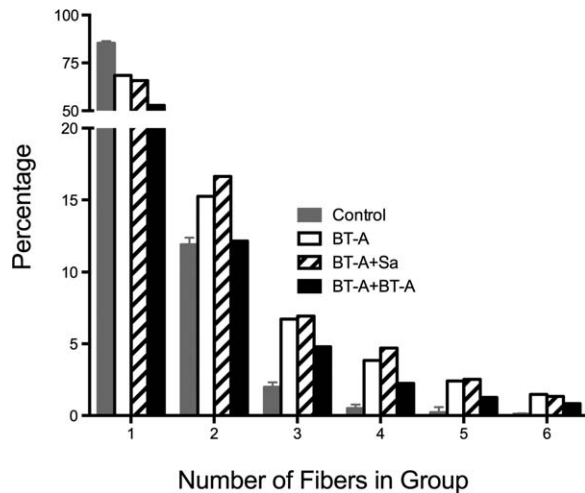


FIGURE 4. Histogram of percentage of type 1 fibers surrounded by type 2 fibers. Note that in control skeletal muscles, $85 \pm 1\%$ of type 1 fibers were completely surrounded by type 2 fibers (i.e., number of fibers = 1) while in all 3 of the BoNT groups, less than 70% of type 1 fibers were completely surrounded by type 2 fibers ($P < 0.001$). Data are shown only for “groups” up to 6 fibers, which was the limit of control muscles. Several BoNT injected muscles contained fiber groups of over 20 fibers.

suggested from clinical experience. We suspect and have previously reported,^{9,10} that a relatively low level of muscle force production can still result in enough function to produce a functional effect that would result in the need for treatment. Thus, even after 3 months, when muscle function is approximately half of normal, spastic activity of this muscle may still create joint deformity requiring treatment. We thus do not interpret our animal model results as contradicting clinical experience. Rather, we believe these results uncover a previously unappreciated phenomenon. When muscles are injected with BoNT before complete recovery, the subsequent BoNT effect is much stronger compared with the initial effect. We injected 6 U/kg at both time intervals, but, based on our understanding of the relationship between dose and efficacy in this animal model (see Fig. 1A of Hulst et al. 2013), we suggest that a dose of less than half may be as efficacious as the initial injection. Clearly our results suggest that even when injections are separated by 3 months, the muscle has not fully recovered in terms of either structure (Figs. 2B,C) or function (Fig. 2A).

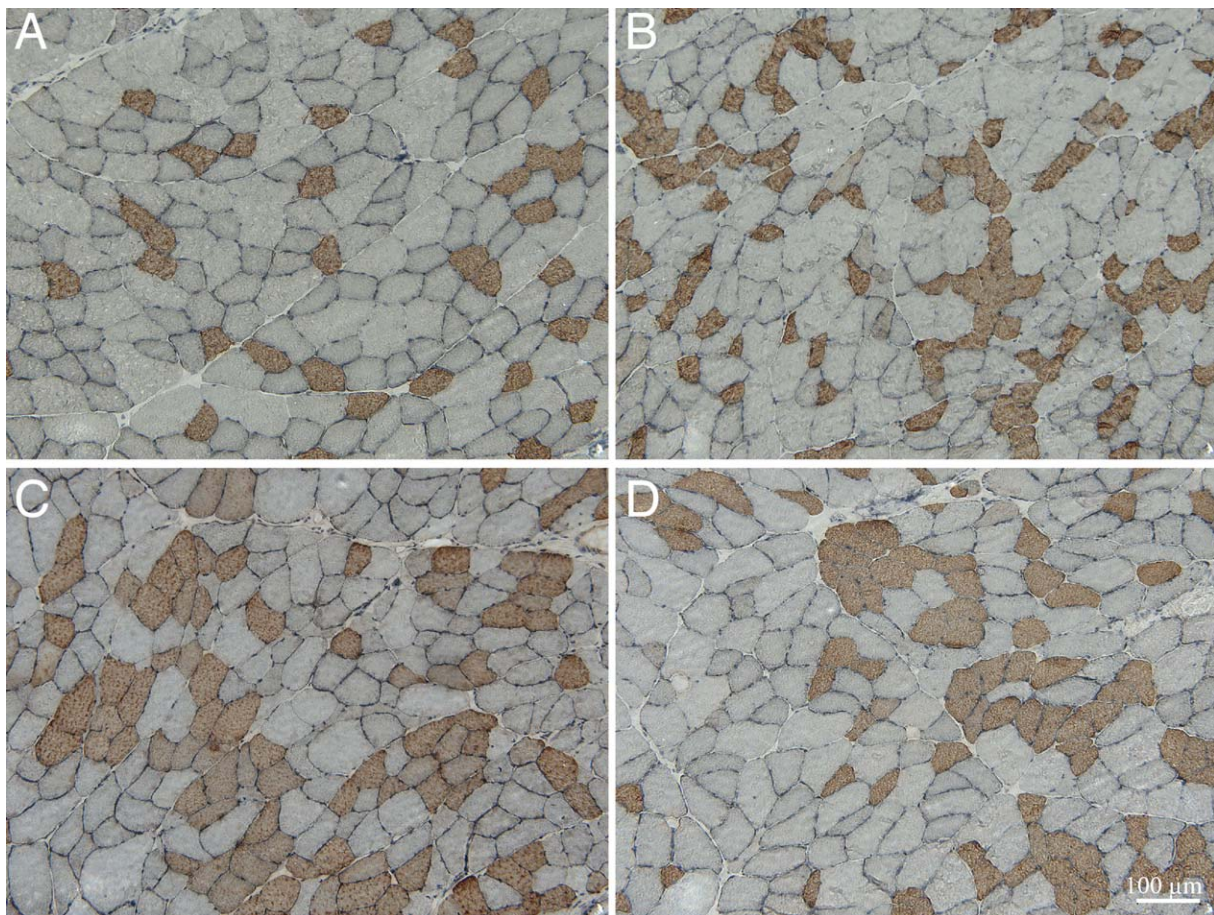


FIGURE 5. Representative immunohistochemical images from contralateral control of BoNT + Saline injected (A), BoNT injected (B), BoNT + BoNT (C), or BoNT + Saline (D) groups. All sections were labeled with a primary antibody against type 1 myosin heavy chain. Fiber type grouping was observed in all BoNT injected muscles, with groups ranging from 16–24 fibers. Scale bar = 100 μm .

Again, this appears to conflict with traditional clinical wisdom which suggests that higher doses are required upon subsequent injections. It should be noted that our studies were performed in normal rat muscle, and therapeutic use of BoNT is typically performed on muscles with neuromuscular abnormalities, so it is not clear how well our results apply to abnormal muscle.

As with our previous studies,^{9,10} there was a highly significant relationship between muscle fiber cross-sectional area and joint torque in all muscles studied (data not shown). Linear regression between muscle fiber CSA and joint torque revealed a highly significant result ($P < 0.0001$) with the relationship described by the equation:

$$\text{Torque (Nm)} = 2.6 * 10^{-5} \text{ Area } (\mu\text{m}^2) - 0.03 \quad (n=30, r^2=0.71)$$

This is nearly the identical regression relationship reported previously.¹⁰ The significance of this finding is that muscle fiber area appears to be the primary factor producing joint torque at both the 3- and 6-month postinjection time points. The BoNT-induced muscle fiber atrophy secondary to interruption of NMJ activity appears to be responsible for the muscle functional deficit. This is consistent with observed changes in human skeletal muscle after BoNT injection.¹⁷ Because the torque-muscle fiber area relationship still holds after a second injection, we suggest that the NMJs are “weakened” by the initial injection so that subsequent injections disrupt the NMJ interface to a much greater extent compared with when the NMJ is healthy.

Increased Collagen Content. We measured “fibrosis” indirectly using the hydroxyproline assay, because alterations in fiber size that occur in atrophy models often appear as fibrotic when actually the decreased fiber size allows expansion of the extracellular space. While the collagen content increases observed in injected legs were expected, contralateral increases were not. Our interpretation is that BoNT-induced muscle fiber atrophy and recovery leads to collagen deposition resulting in increased collagen content in the 2 injected muscles. However, the BoNT+BoNT and BoNT+Saline groups are also 3 months older than the single BoNT group. There is ample evidence that age and connective tissue content are correlated positively, which leads us to interpret the contralateral effect as an age effect.

Lipid Accumulation. This report of increased lipid accumulation after BoNT injection is consistent with previous reports in both rabbit and mouse

models, thus it does not appear that this result is species specific.^{7,8} In these previous studies, the total unit of injection was 21 U/kg in rabbits over a period of 6 months⁷ and approximately 0.6 U/g in mice over a 56-day period.⁸ Lipid accumulation in those studies was qualitatively much more severe (but was not quantified), so there does seem to be a cumulative dose-response relationship. We do not believe that the increased intramuscular lipid accumulation is caused by a direct effect of BoNT; rather, it appears to be an indirect result of NMJ paralysis. This theory is based on previous studies that have demonstrated accumulation of intramuscular lipid after denervation,^{18,19} and that small BoNT doses do not result in lipid accumulation.¹⁰

The mechanisms involved in intramuscular lipid accumulation are not completely known, but it has been hypothesized that satellite cells themselves have adipogenic potential.²⁰ It is also possible that the recently described “fibro-adipogenic progenitors” identified in skeletal muscle^{21,22} are directly or indirectly activated by BoNT treatment. Future studies using lineage tracing are required to determine the precise source of intracellular lipid deposition and its relationship to BoNT injection. It is also possible that muscle paralysis may lead to increased storage of energy-producing substrates. Lipid is the main energetic substrate of type 1 and type 2A fibers, and the double injected rat TA muscle has nearly 50% of these fiber types (Table 2), compared with less than 5% in the normal rat TA.²³ A previous report demonstrating a significant increase in lipoprotein lipase (an enzyme that facilitates transport of free fatty acids into muscle) after BoNT injection may be causally related to the lipid accumulation.²⁴ Of course, it is also possible that an increase in type 1 and type 2A fibers, along with increased lipid actually results in a less-fatigable muscle. This result that might be clinically preferable, because it has been reported that children with cerebral palsy suffer from increased muscle fatigue,²⁵ but this observation is not universal.²⁶

Muscle Fiber Type Transition. It is well known that skeletal muscle fibers are dynamic structures capable of altering their phenotypes due to altered neuromuscular activity.²⁷ In general, transition toward the faster muscle fiber phenotype is observed after decreased muscle use, such as immobilization, spinal cord injury, and weightlessness, while transition toward the slower fiber phenotype is observed with increased loading (exercise, chronic stretch, functional overload, and hypertrophy).¹⁵ Although BoNT injection caused temporary muscle denervation and resulting decreased neuromuscular activity, we measured a

higher expression of type 1 (slow muscle fibers) and type 2A (fast oxidative muscle fibers) myosin heavy chains in injected muscles, which is opposite to results from other disuse models but is in agreement with previous studies in the rat gastrocnemius, plantaris,²⁸ laryngeal,²⁹ and extraocular muscles³⁰ after BoNT injection. This result reinforces the idea that denervation and, in this case, chemodenervation, elicits a muscle response that is not simply related to muscle “use level” but may also be related to NMJ activity and/or neurotrophic factors.

In terms of fiber type distribution, muscle cross-sections are described as having a mosaic pattern of fiber types.^{31,32} While fibers of the same type are often adjacent in normal rat TA muscle, 97% of the time fibers were either isolated (85% of the fibers) or in “groups” of 2 (12% of the time; Fig. 4, open bar). We never observed more than 6 fibers adjacent to each other in normal muscle. In contrast, in any of the BoNT groups, isolated fibers or double fibers were only observed 74–85% of the time (Fig. 4, black, hatched, and gray bars). In other words, approximately 15% of the fibers in the BoNT groups appeared to be “grouped” compared with normal muscle fibers. While this may not have a functional effect, nearly 15% of the fibers being grouped suggests that, following chemodenervation, sprouted axons innervate adjacent fibers and do not reinnervate the original NMJ. Therefore, the process of chemodenervation does not appear to be entirely reversible anatomically. This finding does not necessarily contradict the classic paper demonstrating reinnervation at the original NMJ,³³ because our results do not eliminate the possibility of reinnervation at the original NMJ, but only demonstrate that it is not a universal phenomenon. Also, our method only investigated type 1 muscle fibers, which represent less than 0.5% of the fibers in rat TA muscle. The reinnervation status of the remaining 99.5% of type 2 fibers was not determined in this study. Of interest, despite the increase in type 1 myosin heavy chain content measured in the injected muscles (Table 2), there was no parallel increase in the number of type 1 fibers measured in the muscle. These data are not necessarily contradictory. Because muscle fibers can express multiple myosin heavy chain isoforms, protein gel analysis of myosin content is not necessarily reflected in the immunohistochemical type of the fiber.

In summary, these experiments demonstrate that a second identical injection of BoNT 3 months after a previous injection causes a profound and persistent loss in muscle function and altered muscle structure. Structural abnormalities include fiber type transition, fiber type grouping,

and lipid accumulation. The precise mechanisms for these changes are not clear.

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