SYSTEMATIC TEST OF NEUROTOXIN DOSE AND VOLUME ON MUSCLE FUNCTION IN A RAT MODEL

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ABSTRACT: *Introduction*: Onabotulinum toxin serotype A (BT-A) is used for a variety of motor and sensory disorders related to abnormal muscle activity. *Methods*: We developed a high-resolution rodent model to allow precise determination of the effect of BT-A dose (measured in units) and injectate volume (measured in μl) on the efficacy of the injection and systemic side effects. Dorsiflexion is the best indicator of injected and contralateral muscle function. *Results*: One month after injection, dorsiflexion torque of BT-A-injected limbs was decreased significantly in all experimental groups compared with saline controls ($P < 0.05$). Torque was also compared among the BT-A groups, which demonstrated a significant effect of dose ($P < 0.001$), but no effect of volume ($P > 0.2$) and no dose $\times$ volume interaction ($P > 0.3$). Similar results were observed for other parameters measured. *Conclusions*: These data demonstrate that injection dose and not volume or concentration is the primary determinant of neurotoxin efficacy in a rodent model.

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The therapeutic uses of onabotulinum toxin serotype A (BT-A) have expanded rapidly to include treatment for many motor and sensory disorders related to abnormal muscle contraction. A growing body of literature describes the use of this neurotoxin to treat spasticity, often described as a velocity-dependent increased resistance to passive muscle stretch.[1,2] Common causes of spasticity include strokes, multiple sclerosis, traumatic head injury, and cerebral palsy.

Whereas mild spasticity may cause varying degrees of muscle stiffness, severe spasticity often leads to intractable muscle contractures that cause posture and joint deformities.[3] In children, traditional treatment for spastic cerebral palsy includes physical therapy, orthoses, serial casting, intramuscular BT-A, and, should these conservative treatments not resolve the deformity, orthopedic surgery.[4] BT-A has been shown to improve gait mechanics,[5] pain management,[6] range of motion,[7] and clinical outcome scores in affected individuals.[8,9] However, some detrimental side effects have been attributed to BT-A therapy. These include adjacent and distal muscle weakness,[10] and, very rarely, antibody formation.[11] Thus, to minimize undesirable side effects, as well as minimize cost, issues of optimal dosing have become important to practitioners who use BT-A.

Unfortunately, despite the impetus for precise dosing, no standardized guidelines exist for BT-A administration. In the case of spasticity, dosing and dilution recommendations are based on clinical experience and vary widely for a given target muscle. Methods for assaying toxin efficacy also vary widely from the use of electromyographic and clinical measurements in humans[12] to the use of structural and functional measurements in animal models.[13,14] The explicit effects of toxin dose and diluent volume on ensuing chemical denervation and muscle function have not been defined clearly. *A priori*, one could argue that dose or volume, or an interaction between the 2 may affect neuromuscular function due to specific muscle anatomy, neuromuscular junction location, and injectate flow throughout the tissue. For example, if the neuromuscular junctions are not located focally within muscle, it may be important to use a larger volume to facilitate toxin distribution, especially if the dose is small. While previous studies have suggested that toxin dose[12,15] or toxin volume[14,15] affect efficacy, none of these experimental designs was fully crossed and thus, interaction between dose and volume could not be determined.

We described previously a high-resolution rat model to study the effects of BT-A on the tibialis anterior (TA) muscle.[16] This system permits explicit determination of the effects of neurotoxin on muscle structure and function as separate entities. This is superior to generic assays of "neuromuscular function" that cannot account for the anatomical or structural basis for specific effects observed. Therefore, the purpose of this study was to quantify the effects of varying injection dose and

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Abbreviations: ANOVA, analysis of variance; BT-A, onabotulinum toxin serotype A; NMJs, neuromuscular junctions; TA, tibialis anterior

Key words: Onabotulinum toxin; myopathy; spasticity; cerebral palsy; fibrosis

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volume in a fully crossed experimental design, on the functional and structural properties of injected and contralateral muscles to define the therapeutic effect as well as the systemic side effects of this treatment.

MATERIALS AND METHODS

Animal Subjects. Laboratory animals were untrained, mature, male Sprague-Dawley rats (Harlan, Indianapolis, IN) with an average size of 393 ± 16 g (mean $\pm$ SD, $n = 67$, approximate age 5 weeks). Rats were housed 2 per cage at 20–23°C with a 12:12 h dark-light cycle. All procedures were approved by the University of California and the VASDHS Committees on the Use of Animal Subjects in Research. After terminal experiments, animals were euthanized with an intracardiac injection of pentobarbitol sodium (0.5 ml of 390 mg/ml solution).

Experimental Model. Animal subjects were randomly divided into 9 experimental groups. Each group was subjected to a different dose or volume of BT-A (Botox, Allergan, Irvine, CA) injection: 6 units/kg in a 100 μl volume (6 U/100 μl, $n = 10$), 6 U/20 μl ($n = 5$), 6 U/4 μl ($n = 6$), 3 U/100 μl ($n = 6$), 3 U/20 μl ($n = 7$), 3 U/4 μl ($n = 7$), 1 U/100 μl ($n = 6$), 1 U/20 μl ($n = 6$), and 1 U/4 μl ($n = 10$). A tenth group of animals received saline injections to serve as controls for the anesthesia, handling, and injection procedures (CTL, $n = 4$). Thus, in this experimental design, dose was varied over a 6-fold range, while volume was altered over a 25-fold range. Based on the concentration calculation of dose/volume, the design also varied concentration over a 150-fold range.

After anesthesia induction (2% isoflurane, 2.0 L/min), ankle isometric dorsiflexion torque was measured before injection as described previously.[17] Briefly, dorsiflexors were activated (15 V stimulus, 0.3 ms pulse duration, 650 ms train duration) by means of the common fibular nerve, while torque was measured using a custom-designed dynamometer. The site of stimulation was sufficiently proximal to the anterior compartment muscles and the pulse duration sufficiently short to ensure that direct muscle fiber stimulation did not occur. To ensure that an intact neuromuscular unit was being tested, the normal neural recruitment pattern (increasing torque with increasing stimulation intensity) and force-frequency behavior (increasing torque with increasing stimulation frequency) were observed before injection. 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 been shown to have a coefficient of variation of less than 10%[17,18] thus enabling resolution of small changes in dorsiflexor function. After initial torque determination, rats received a one-time BT-A injection into the midbelly of the TA muscle. Pilot experiments using dye injections and neuromuscular junction labeling yielded a distribution of TA neuromuscular junctions concentrated in the proximal 20% of the muscle belly and extending to over 75% of the muscle length.[19] This point was exposed by direct visualization of the muscle belly through an anterolateral longitudinal incision, and approximately half of the injectate was placed slightly distal to this point, the needle was withdrawn slightly (but did not withdraw outside the muscle), and the remainder of the injectate was placed slightly proximal to this point. No egress of injectate was observed. This procedure typically saturated a $\sim$2 mm length of the muscle and created a highly reproducible effect. Because the purpose of this study was to quantify the effects of dose and volume on muscle function and to explicitly exclude injection technique, the method was standardized in this way. Based on the extremely low coefficients of variation observed within groups and very high variation between groups (especially with respect to dose; see below), we are confident that the injection method was not a factor in this experimental design. The volume was administered by the same physician into the midbelly. A 100-μl volume of 0.9% NaCl solution was injected in the same manner into the control animal TA muscles. None of the contralateral muscles were injected.

One month later, experimental and contralateral limb dorsiflexion torque were measured on the same animals using identical procedures. Animals were then euthanized, and bilateral TA 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. For 4 to 5 animals per group, muscle cross-sections (10 μm thick) were taken from the 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 1:1,000) and then with the secondary antibody, Alexa Fluor 594 goat anti-rabbit IgG (Life Technologies, 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

Table 1. Injected and contralateral tibialis anterior muscle masses.*

Injected muscle injectate	Injected muscle mass (g)	Contralateral uninjected muscle mass (g)	*P*-value
BT-A 6 U/100 μl	.475±.025	.928±.015	<0.001
BT-A 6 U/20 μl	.437±.023	.958±.044	<0.001
BT-A 6 U/4 μl	.486±.020	.877±.024	<0.001
BT-A 3 U/100 μl	.531±.054	.945±.021	<0.001
BT-A 3 U/20 μl	.488±.040	.941±.035	<0.001
BT-A 3 U/4 μl	.463±.022	.978±.025	<0.001
BT-A 1 U/100 μl	.804±.066	1.02±.029	>0.1
BT-A 1 U/20 μl	.804±.079	.994±.031	>0.1
BT-A 1 U/4 μl	.605±.027	.939±.019	<0.05
100 μl of saline	.942±.036	.895±.029	>0.9

*N = 5–10/group (see Materials and Methods section). Values represent mean ± SEM.

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.[16] Based on these same pilot experiments, every second field of view in the BT-A injected muscles was imaged. Stereological principles[20,21] were then used, whereby fiber area variance was compared with number of fields measured for accurate determination of fiber area. In this way, the total number of fields required for quantification from each muscle sample was calculated. Six images were required and selected from the images taken from saline-injected and contralateral muscle with the use of a random number generator for fiber cross-sectional area analysis. In the case of the BT-A injected muscles (due to higher fiber area coefficient of variation), 12 images were required and selected with the random number generator. Before analysis, each image was inspected, and areas with obvious sectioning artifacts, large blood vessels, merged fibers, or poor staining quality were omitted.

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 μm^2 or above 14,000 μm^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 were excluded to prevent inclusion of fibers that were sectioned obliquely. Oblique sectioning has the effect of artifactually increasing fiber area.[21]

Collagen Content. Traditional methods of expressing "fibrosis" in terms of 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 BT-A on connective tissue, we estimated the amount of extracellular matrix material based on total collagen content. The value was calculated by measuring hydroxyproline content.[22] 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 3 aliquots of each sample at 550 nm. Hydroxyproline content was converted to collagen content using the extinction coefficient for hydroxyproline and dividing by the number of hydroxyproline residues in a molecule of collagen.

Statistical Analysis. Experimental results were analyzed by one- or two-way analysis of variance (ANOVA) with repeated measures. *Post hoc* Tukey tests were used to compare dependent variables among various pairs of groups. All results are reported as mean ± SEM unless otherwise noted.

RESULTS

The simplest measure of muscle condition after injection, muscle mass, showed that mass of the toxin-injected muscles was decreased significantly in all experimental groups compared with contralateral controls except in the 1 U/100 μl and 1 U/20 μl groups (Table 1). No significant difference in muscle mass was found between the saline injected and contralateral control limb ($P > 0.9$, Table 1). Two-way ANOVA revealed both that injection dose had a significant effect on mass ($P < 0.05$), and there was a significant interaction between dose and volume ($P < 0.05$). Reduction in TA mass relative to saline controls was ~50% for all of the 3 U and 6 U groups. The 1 U/4 μl group demonstrated a 35% reduction, while the 1 U/100 μl and 1 U/20 μl groups had only ~15% reduction in mass. Contralateral TA muscle masses

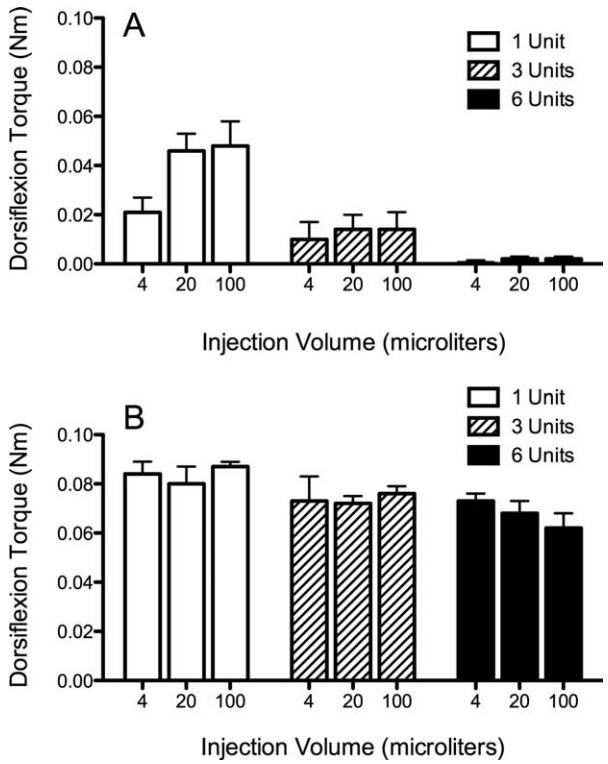

FIGURE 1. (A) Dorsiflexion torque measured from BT-A-injected groups by volume (1 U: white bars, 3 U: hatched bars, 6 U: black bars). Two-way ANOVA revealed a significant effect of dose, but no significant effect of volume or interaction. Dotted line represents the average contralateral limb torque. **(B)** Dorsiflexion torque of contralateral limbs. *-represents significant difference between volumes within a dose.

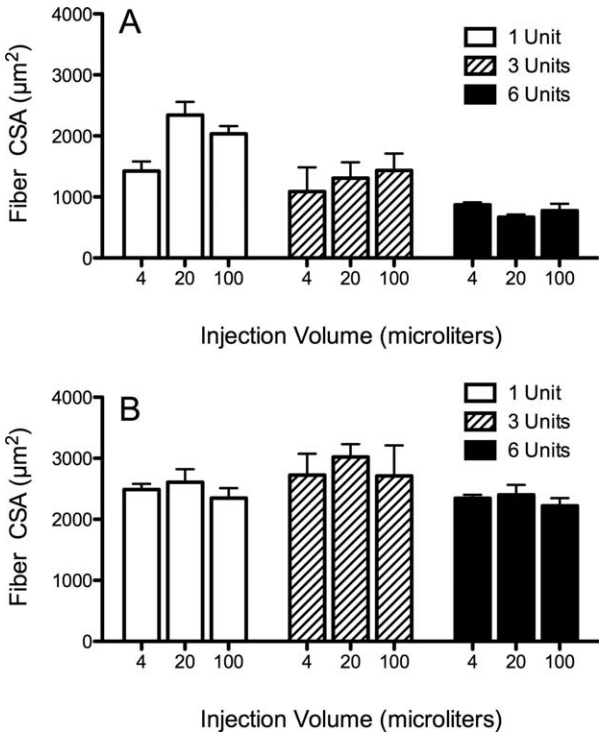

FIGURE 2. (A) Muscle fiber cross-sectional area measured from BT-A-injected groups by volume (1 U: white bars, 3 U: hatched bars, 6 U: black bars). **(B)** Muscle fiber cross-sectional area measured from the contralateral limbs. Two-way ANOVA revealed a significant effect of dose, a significant effect of volume, and significant interaction.

were similar to saline control values, revealing a negligible systemic effect ($P > 0.5$).

Unfortunately, muscle mass can be a poor predictor of function, because mass can change due to tissue edema, inflammation, fibrosis, and muscle length change. Thus, our most meaningful functional predictor, dorsiflexion torque was the parameter of focus. Postinjection dorsiflexion torque of BT-A-injected limbs was decreased significantly in all experimental groups compared with saline controls ($P < 0.05$, Fig. 1A). Torque was also compared among the BT-A groups, which demonstrated a significant effect of dose ($P < 0.05$) but no effect of volume ($P > 0.2$) and no interaction between dose and volume ($P > 0.2$). Compared with saline controls, all 6 U groups demonstrated an ~95% reduction in torque, while the 3 U groups had an ~80% reduction. The 1 U/100 μl and 1 U/20 μl groups demonstrated an ~35% reduction, while the 1 U/4 μl group had a 65% reduction. We also observed a significant effect of dose ($P < 0.005$) but no significant effect of volume ($P > 0.7$) or dose × volume interaction ($P > 0.5$) in contralateral limbs (Fig. 1B). There was no significant difference between contralateral and saline-injected limbs in the saline control group ($P > 0.5$, data not shown).

Because muscle fiber area and muscle force are related directly in normal muscle, we measured fiber area in all experimental samples. Muscle fiber cross-sectional area results roughly paralleled dorsiflexion torque and TA mass. Dose had a significant effect on muscle fiber size (Fig. 2A; $P < 0.05$), and a significant interaction was observed between dose and volume ($P < 0.05$). The volume effect just failed to achieve statistical significance ($P = 0.06$, $\beta = .64$). Reduction in fiber area from contralateral control values was ~75% across the 6 U groups and ~50% across the 3 U groups (Fig. 2B). The 1 U/100 μl and 1 U/20 μl groups demonstrated ~10% and ~20% reductions in fiber size, respectively, while the 1 U/4 μl group had a ~45% reduction. No differences in contralateral limb fiber sizes were found between the experimental and saline control groups ($P > 0.2$, data not shown).

Because we considered the torque values to be representative of fiber area and thus an indicator of muscle fiber force, we regressed fiber area on torque across all experimental groups and found a significant relationship between the 2 variables ($P < 0.0001$) and a fairly linear relationship with the regression equation explaining approximately

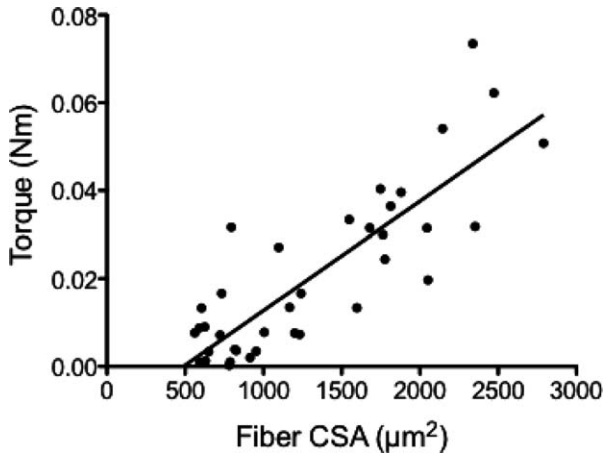

FIGURE 3. Relationship between dorsiflexion torque and muscle fiber cross-sectional area for all animal subjects ($n = 67$). Data vary continuously across the independent variable, demonstrating variability in response even to identical injection paradigms. Data are well described by a line with the equation: Torque (Nm) $= 2.5 \times 10^{-5}$ Area (μm^2) $- 0.017$ ($n = 41$, $P < 0.001$, $r^2 = 0.76$). These results suggest that muscle fiber area is a strong predictor of function 1 month after injection.

75% of the experimental variability ($r^2 = 0.76$, Fig. 3).

There was no overall effect of either dose or volume on collagen content in TA muscles and no significant interaction (Fig. 4; $P > 0.3$ for all factors). All injected muscles had a collagen content that was significantly above the saline-injected muscles (gray line, Fig. 4; $P < 0.05$) with the exception of the 1 U/20 μl group.

DISCUSSION

The purpose of this study was to quantify the relationship between injectate volume and toxin dose systematically in the rat anterior compartment model. The main result of this study was that dose was, by far, the most important predictor of the effect of neurotoxin injection. Neurotoxin dose dramatically decreased dorsiflexion torque (Fig. 1A), muscle mass (Table 1), and muscle fiber area (Fig. 2A). As mentioned above, the dose effect was much stronger than either the volume effect (when observed) or dose $\times$ volume interaction (when observed). The dominance of dose as an intervention is even more impressive in light of the fact that dose was only varied over a 6-fold range (1 U to 6 U), while volume was varied over a 25-fold range (4 μl to 100 μl), and neurotoxin concentration was varied over a 150-fold range (.01 U/μl to 1.5 U/μl). Thus, if anything, our experimental design was biased away from showing the dose effect. The clear dominance of dose as the most significant effect on function appears to indicate that the key factor that determines the efficacy of the BT-A neurotoxin in this model system is the number of active light chain enzymes that are delivered to neuromuscular junctions. The greater the number of enzyme molecules, the greater the number of light chains available to cleave the SNAP-25 SNARE protein. As a result, synaptic vesicles from these axons cannot activate their associated muscle fibers.

Based on the literature and our knowledge of muscle-toxin interaction, it was not obvious *a priori* whether dose, volume, or an interaction between the 2 would have the greatest effect on muscle phenotype and any contralateral effects. This is because predicting which of these factors most strongly affects the muscle depends on the nature of the interaction between the injectate and the muscle tissue. For example, if spreading of the injectate toward neuromuscular junctions (NMJs) were a limiting process, injectate volume would be expected to have a strong effect on injection efficacy due to higher volumes producing a greater effect by increasing muscle pressure and driving the spread of the injectate and the larger carrier volume facilitating transport of the toxin to the presynaptic terminal of the NMJ. A previous study in the mouse hindlimb measured some dose and volume combinations and also suggested that dose was a strong predictor of efficacy.[15] Unfortunately, the design of that study was not fully crossed so that interaction terms could not be defined. In addition, data were presented relative to contralateral muscle force so that systemic effects could likewise not be defined. Based on the relatively modest contralateral effects observed here and because mouse anatomy is homologous to rat anatomy,[23,24] we believe that the animal model literature as a whole suggests that dose is the dominant factor in determining efficacy, and that systemic side effects are relatively small. Thus, whether a unit of toxin is suspended in 1, 25, or 100 μl, the functional effect is approximately the same (Fig. 1A). It would be interesting to determine whether injectate viscosity affects efficacy in light of this finding. The only functional side-effect we were able to detect was a slight decrease in contralateral torque at high toxin doses and volumes (Fig. 1B). This result probably reflects leakage of toxin molecules out of the injected muscle into the circulation with systemic distribution. However, recent studies suggest that contralateral effects may be mediated centrally, so the mechanism of this finding is subject to further study.

The fact that dose $\times$ volume interactions were either absent or relatively small demonstrates that the 150-fold variation in neurotoxin concentration does not affect efficacy strongly. This is probably a reflection of the extremely high affinity between the neurotoxin molecule and its presynaptic receptor.[25] Increasing concentration does not tend to

"drive" the toxin to the receptor as would be expected based on first- or second-order receptor-ligand interaction of low affinity.[26] We conclude that the functional binding capacity of the receptor is saturated at a concentration of only 0.01 U/μl.

Our experimental results are consistent with a great deal of the neurotoxin literature. Differences between studies appear to relate largely to the different model systems used, different muscles injected, and different measures of injection efficacy. For example, Shaari and Sanders showed that both dose and volume were strong predictors of efficacy using muscle fiber glycogen staining as a measure of efficacy.[27] However, muscle glycogen content varies significantly between fiber types and even between muscles,[28] requiring quantitative image processing to produce truly objective results.[29] Thus, this result may reflect intrinsic glycogen content differences among muscles in addition to or in opposition to the neurotoxin effect.

Most clinical studies are at least consistent with our conclusion that injection volume does not play a large effect in determining neurotoxin efficacy, while the number of units has a large effect. However, it is very problematic to compare clinical studies with our animal model system. This is not only due to the fact that we are studying small muscles in an animal model, but also that, typically either dose or volume were tested in isolation making it impossible to define an interaction (if any) between these 2 factors.

Many have shown significant effects while others have shown no significant effect of injection volume on clinical outcome.[30–32] In a randomized controlled trial, dose and volume were altered, and their effect on dynamic forehead lines was defined. The authors describe an interaction between dose and volume but the data were not analyzed using a statistical method that explicitly calculates interaction terms.[33]

While we attempted to scale doses and volumes studied to those that would be relevant to human studies, our work clearly has several limitations. First and foremost, the rat anterior compartment is relatively simple architecturally compared with almost all human muscles.[24,34] This, partly by virtue of the smaller size and partly due to the slightly lower connective tissue density, could facilitate diffusion throughout the muscle. Thus, extrapolation of these results to other rat or human muscles in general should not be made. Second, the lowest volume we could reliably inject with a Hamilton syringe was 1 μl, which is still approximately 0.1% of the TA volume.[24] Much smaller relative volumes can be injected into large human muscles, and it is possible that these smaller volumes would enable teasing out a volume effect.

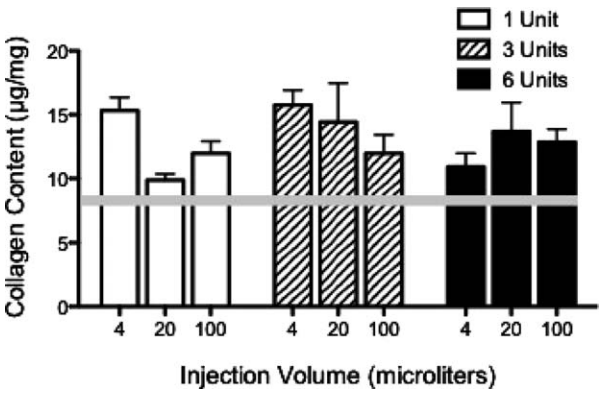

FIGURE 4. TA muscle collagen content from BT-A-injected groups by volume (1 U: white bars, 3 U: hatched bars, 6 U: black bars). Two-way ANOVA revealed no significant effect of dose or volume on collagen content and no significant interaction. Grey bar represents the lumped average of all contralateral control muscles (8.75 ± 0.61 μg/mg, $n = 55$).

However, we believe that our data indicate that there is a relatively accessible and uniform distribution of motor endplates in the TA, which facilitates "hitting" the NMJ targets with injections. The extent to which the same results would be obtained in muscles with different architectural properties or distributions of NMJs is not known. However, in a randomized controlled trial, it was demonstrated that targeted injections were more efficacious compared with low volume, random injections, so it appears that the concept of NMJ targeting has validity in patients as well.[35] Because the volume of the rat TA is approximately 1 ml, our injectate volumes represent 0.1% to 10% of the muscle volume. It is possible that some of the systemic effect, especially at high volumes is due to diffusion of toxin out of the muscle. It must also be mentioned that these data are applicable only to this neurotoxin, and application to other products remains to be established. This is due in part to the different product formulations and also to the known differences in clinical efficacy and safety profiles among products. Also, experimental data and the U.S. Food and Drug Administration official position is that units from 1 product are neither interchangeable nor convertible among products.

There was no systematic effect of toxin dose or volume on collagen content on TA muscles (Fig. 4). There has been no previous systematic study of these effects on collagen content in muscle. We suspected that higher toxin concentrations might cause an inflammatory reaction, which is known to lead to fibrosis in muscle, probably by means of the transforming growth factor-β pathway.[36,37] However, this was not the case. While collagen levels were increased by 10–100%, there was no obvious relationship to any of the experimental parameters in our design. Because injection

efficacy, and therefore, degree of denervation, varied widely among the injected groups (Fig. 1A), these data strongly suggest that denervation *per se* does not activate the fibrosis pathway itself. Further studies are required to define the actual stimulus for this response.

Finally, the quantitative measures of dorsiflexion torque and muscle fiber cross-sectional area are direct reflections of the muscle tissue without consideration of motor control, neural drive, or NMJ function that would normally affect interpretation of the functional results of clinical studies. Future studies are required to define the extent to which these findings are applicable to specific injections of human skeletal muscles.

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