Dose- and Volume Dependent-Response to Intramuscular Injection of Botulinum Neurotoxin-A Optimizes Muscle Force Decrement in Mice

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ABSTRACT: Botulinum neurotoxin-A (BoNTA) is a potent neurotoxin used to alter muscle tone to manage spasticity and to provide tendon bioprotection; however, the appropriate dose and injection volume to administer is not defined. Male mice ($n = 120$) received BoNTA injections into one gastrocnemius with either a constant volume (10 μl) with a variable dose (1, 3, 6 U/kg) or a constant dose (3 U/kg) in a variable volume (2.5, 5, 10, 20, 30 μl). Electromyographic (EMG) examination, muscle force generation (MFG), and wet muscle mass were measured in the ipsilateral and contralateral limbs at 1, 2, 4, or 12 weeks post-injection. MFG and EMG responses decreased to approximately 40% of contralateral after a 1 U/kg injection and 0% of contralateral by 3 and 6 U/kg injection at 1 week after injection. Neuromuscular blockade was greatest with a 10 μl injection volume. MFG, EMG examination, and wet muscle mass reached contralateral values 12 weeks after injection for all injection doses and volumes tested. Effective injection doses and volumes were identified for producing full and partial neuromuscular blockade in the mouse gastrocnemius. These findings have important clinical implications in the intramuscular administration of BoNTA to manage muscle tone. J Orthop Res

Keywords: botulinum toxin; muscle force; mouse; dose; volume

Botulinum neurotoxin-A (BoNTA) is a potent toxin with high specificity for the neuromuscular junction.[1] Chemodenervation with BoNTA has important clinical uses in the management of neuromuscular disorders including achalasia, hyperhydrosis, and spasticity and an emerging role as tendon bioprotection.[2–6] Although many clinicians administer BoNTA injections, little consensus exists regarding the appropriate injection dose and volume.[2,7,8] Clinical preparations for BoNTA intramuscular injection are most commonly in concentrations of 50 and 100 U/ml with an injection volume of 2–4 ml, but may be as concentrated as 500 U/ml and injected in volumes of up to 8 ml.[2,7,9] Furthermore, the dose administered to reach clinical goals in muscle tone is highly variable.[7–9]

Endocytotic uptake of BoNTA by the distal neuron results in a conformational change in the light chain with subsequent proteolytic cleavage of the vesicle docking protein, SNAP-25.[1,10] Cleavage of SNAP-25 prevents vesicular release of acetylcholine into the synaptic cleft of the neuromuscular junction causing muscle paralysis. The effects of BoNTA injection may be seen within 8 h but the blockade is temporary.[1,11–13] Nerve growth factors, myonuclear addition, axonal sprouting, and motor end plate activity are increased within a week.[14–17] Muscle recover occurs within 3–6 months in humans which enables BoNTA to be a valuable tool in reversibly altering muscle tone.[8,18]

Literature to date offers little information on a systematic approach to the effects of dose and volume on the neuromuscular physiology following intramuscular administration of the toxin.[6–9] Detailed evaluation of BoNTA injections and neuromuscular physiology with an animal model would provide valuable insight into dosing techniques for the clinical administration of BoNTA. A murine model was used to test the hypothesis that the degree of muscle inhibition can be optimized by delivering the appropriate dose and volume to the targeted muscle through alteration of the independent variables, BoNTA dose and injection volume, as assessed by outcome measures of electromyographic (EMG) examination, muscle force generation (MFG), and wet muscle mass.

MATERIALS AND METHODS

The protocol was approved by the Wake Forest University Animal Care and Use Committee. Mice (male CD1, Charles River Laboratories) were housed in an animal resources facility with rat chow and water provided ad libitum.

Botulinum Toxin Injection

A single bottle of lyophilized botulinum neurotoxin type-A (BOTOX, 100 U; Allergan, Irvine, CA) was reconstituted in 1.0 ml of 0.9% saline. Aliquots of 50 μl were frozen in liquid nitrogen and stored at −80°C. As previously reported, freezing the toxin caused no decrease in efficacy in intramuscular injections.[19] Aliquots were retrieved, thawed, and diluted with 0.9% saline to the appropriate injection volume.

Mice were weighed to verify the weight range prior to anesthesia with isoflurane (IsoFlo, Abbott Laboratories, North Chicago, IL). Five mice per group per time point ($n = 140$ mice at 23 ± 1 g) were injected as previously described[19] with the following modifications. Injections were performed by the same person (A.V.S.) under a dissecting microscope (Wild-Heerbrugg M650, Gais, Switzerland) using a 30 gauge needle attached to a 10, 25, or 50 μl Hamilton syringe based on the injection volume. For dose studies, a dose of 1, 3, or 6 U/kg in a constant volume of 10 μl was injected into the gastrocnemius. A dose of 3 U/kg was administered in a total volume of 2.5, 5, 10, 20, or 30 μl for the volume studies. To ensure maximal inhibition, 50% of the

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total dose volume was injected into each head of the gastrocnemius. The contralateral (control) legs did not receive saline injections; however, the MFG values produced by the control legs in this study were within our previously published control ranges for both the injected and uninjected legs.[19]

Assessment of MFG

Mice were retrieved for testing at 1, 2, 4, and 12 weeks after injection. Mice were weighed and anesthetized using isoflurane during the experiment. EMG examination was conducted according to our previously published methods with the following modifications.[12] The nerve was stimulated with a nonrecurring stimulus (0.8 mA, 0.1 ms duration). EMG recording was performed twice per leg, and the average latency and compound muscle action potential (CMAP) was reported.

Following EMG evaluation, the grastrocnemius was isolated and mice were transferred to a platform for MFG assessment as described.[19] The nerve was stimulated to elicit maximal single twitch response (0.10–2.0 V, 0.5 ms duration) followed by repeated stimulation of varying frequency with constant voltage to elicit tetany (0.950 V; 15–200 Hz). Peak response was attained within 200 ms. The procedure was repeated for the contralateral leg.

Muscle Mass Determination

Following MFG testing, the gastrocnemius was dissected, removed, and trimmed of its tendinous attachments. The muscle was immediately blotted and weighed on a balance (Metter, Toledo, OH). Mass determination was repeated for the contralateral leg. Animals were euthanized under anesthesia at the end of each experiment.

Data were reported as percent contralateral (control) limb. A multivariate analysis of variance (ANOVA) was used to analyze dose, volume, and time effects. When significant main effects were identified, Bonferroni post-tests were used for post hoc tests. A significance level of $p \leq 0.05$ was used for all tests. Error bars indicate standard error of the mean.

RESULTS

EMG Evaluation

Latency was significantly increased in all experimental legs versus control legs (Table 1). Peak latencies occurred 1 week following BoNTA injection for all but the 2-week 6 U/kg dose response, which occurred at week 2. Doses of 3 and 6 U/kg produced significantly greater latencies than the 1 U/kg dose (Table 1). The greatest increases in latency in the volume studies occurred with 10 and 20 μl injection volumes. Maximal efficacy decreased with 2.5, 5, and 30 μl injection volumes (Table 2). Latency was equivalent to contralateral limb values by 12 weeks following injection for all doses.

Table 1. EMG Evaluation of Dose Response to BoNTA in 10 μl Injection Volume

Week	1 U/kg	3 U/kg	6 U/kg
% Control CMAP			
1	76 ± 9	20 ± 3***	18 ± 4***
2	95 ± 3	42 ± 8**	23 ± 3***
4	85 ± 15	60 ± 12*	83 ± 6
12	103 ± 10	95 ± 6	103 ± 13
% Control CMAP latency			
1	115 ± 5	169 ± 16***	142 ± 9*
2	109 ± 3	121 ± 3	160 ± 12**
4	131 ± 4	139 ± 9	135 ± 6
12	110 ± 4	98 ± 5	102 ± 3

***$p < 0.0001$, **$p < 0.01$, *$p < 0.05$ versus 1 U/kg dose of same time point.

CMAPs values were inversely proportional to the latency values, as expected, with peak decrements in amplitude generally seen at 1 week post-injection (Tables 1 and 2). Doses of 3 and 6 U/kg decreased CMAP the most ($p < 0.0001$ vs. 1 U/kg), while a 1 U/kg dose decreased CMAPs by 25% (Table 1). An injection volume of 10 μl was the most effective in reducing amplitude response ($p < 0.0001$ vs. 2.5 and 30 μl), but was not significantly different from 5 or 20 μl (Table 2). The decrement in CMAP for 2.5 and 30 μl injection volumes was small (~15%), which was significantly less than the respective values for 5 μl ($p = 0.0024$, $p = 0.0002$) and 20 μl ($p = 0.0154$, $p = 0.0012$). All groups demonstrated a clear trend toward recovery by 12 weeks post-injection.

MFG Evaluation

MFG responded to BoNTA injection with dose-dependent decrements similar to EMG responses. MFG decreased 60% with 1 U/kg dose and was effectively abolished by 3 and 6 U/kg doses (Fig. 1). Force recovered to 40–60% contralateral force by 4 weeks post-injection, and MFG recovered to approximately 100% of contralateral values by 12 weeks for all doses tested in both single twitch and titanic stimulation (Fig. 1).

MFG testing suggests that the most effective volume for injection was 10 μl, which abolished MFG in both single twitch and 100 Hz tetanic stimulation 1 week after injection (Fig. 2a). Partial inhibition of MFG was achieved by altering injection volumes. Volumes of 5 and 20 μl decreased single twitch MFG by 75%, while 2.5 and 30 μl reduced MFG by 33%. Volume response in tetanic stimulation produced similar decrements in MFG (Fig. 2).

Muscle force recovered incrementally and reached complete or near complete recovery by 12 weeks post-injection (Figs. 1 and 2). All injection volumes other than 30 μl exhibited recovery to approximately 35% control after 2 weeks, 60–80% of contralateral force by 4 weeks, and near full recovery by 12 weeks post-injection (Fig. 2).

Muscle Mass

Gastrocnemius muscle mass decreased after the administration of BoNTA, but in all groups, muscle mass recovered to contralateral mass by 12 weeks post-injection (Table 3). Peak atrophy occurred at 4 weeks post-injection with 3 and 6 U/kg groups showing a mass reduction to about 60% of contralateral

Table 2. EMG Evaluation following 3 U/kg BoNTA Injection in Varying Volumes

Week	2.5 μl	5 μl	10 μl	20 μl	30 μl
% Control CMAP					
1	70 ± 3	37 ± 1[b]	20 ± 3[a]	44 ± 7[c]	85 ± 7
2	73 ± 13	51 ± 6	42 ± 8	55 ± 10	50 ± 5
4	57 ± 6	80 ± 8	60 ± 12	61 ± 11	41 ± 5
12	87 ± 7	105 ± 9	95 ± 6	71 ± 5	97 ± 17
% Control CMAP latency					
1	124 ± 4	132 ± 7	169 ± 16[a]	153 ± 7[b]	121 ± 4
2	115 ± 9	117 ± 6	121 ± 3	136 ± 5	117 ± 3
4	121 ± 4	112 ± 5	139 ± 9	128 ± 11	119 ± 5
12	109 ± 3	103 ± 2	98 ± 5	113 ± 4	101 ± 1

[a]$p < 0.0001$ versus 2.5 and 30 μl; [b]$p < 0.01$ versus 2.5 and 30 μl; [c]$p < 0.05$ versus 2.5 and 30 μl.

(Fig. 3a); however, 6 U/kg 4-week mass values were not significantly different from those obtained 1 and 2 weeks after injection. Wet muscle mass loss was significantly less severe in 1 U/kg animals (Fig. 3a).

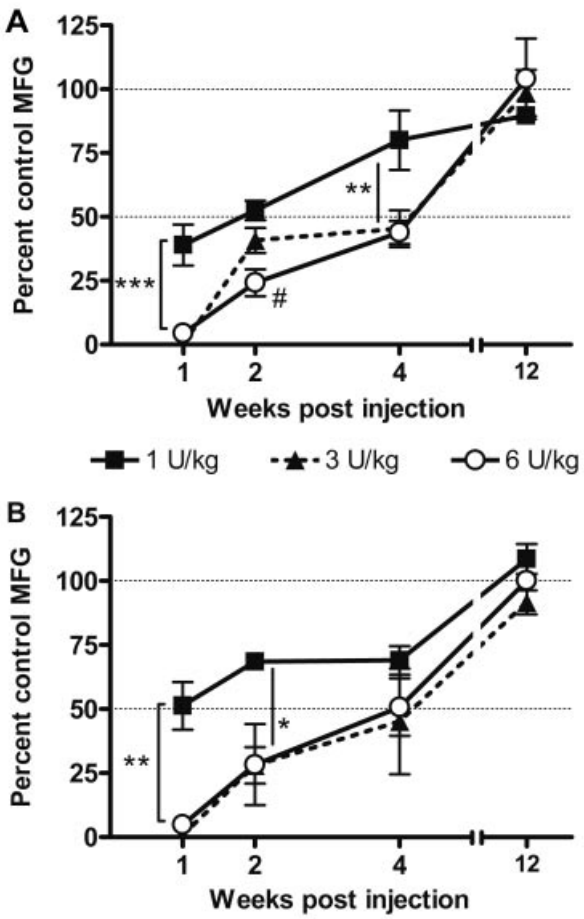

Figure 1. MFG responds in a dose-dependent manner to BoNTA. MFG response was fully inhibited with 3 and 6 U/kg and partially inhibited with 1 U/kg. MFG fully recovered by 12 weeks after injection. (A) Single twitch response. (B) Tetany response (100 Hz stimulation). (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ for 3 and 6 U/kg vs. 1 U/kg at same time point; #$p < 0.05$ for 6 U/kg vs. 1 U/kg at same time point). Control = contralateral limb.

Injection volume affected wet muscle mass in a slightly different time course than that generated by the dose response (Table 3). The 5 and 30 μl injections produced respective peak decrements in contralateral mass at 2 weeks. Peak atrophy occurred at 1 week post-injection in the 20 μl injection group, but the difference between weeks 1 and 2 was statistically insignificant. The 2.5 μl injection group reached peak decrement at 4 weeks post-injection (which was significantly less severe than 10 μl injections, $p < 0.01$). Figure 3b illustrates volume-related responses in peak decrement in wet muscle mass of the mouse gastrocnemius following injection of 3 U/kg BoNTA.

DISCUSSION

Little information is currently available that systematically characterizes the effects of dose and volume of intramuscular BoNTA injection on neuromuscular physiology.[20,21] Previous studies suggest that limited changes in volume of BoNTA intramuscular injections produce little change in efficacy in humans.[22,23] The present study analyzed how injection dose and volume altered the efficacy of intramuscular BoNTA injections. Doses administered in this study fall within clinical recommendations and were previously demonstrated to be effective in the mouse, rat, and rabbit.[8,12,19,20] The results demonstrate a dose-dependent response to BoNTA injection and that injection volume plays a critical role in determining the efficacy of a particular dose.

The EMG evaluation of the gastrocnemius was congruent with MFG results. Increases in latency and decreases in CMAP corresponded with the peak decrements in MFG (Table 1, Figs. 1 and 2). The predictive value of CMAPs for MFG response is not surprising, since BoNTA attenuates vesicular release of acetylcholine at the neuromuscular junction. A low CMAP may indicate low levels of acetylcholine release and an impaired muscle contraction. Signal transmission may recover and the muscle may be capable of depolarization, but muscle contractility may not be restored. CMAPs and latency were not significantly different

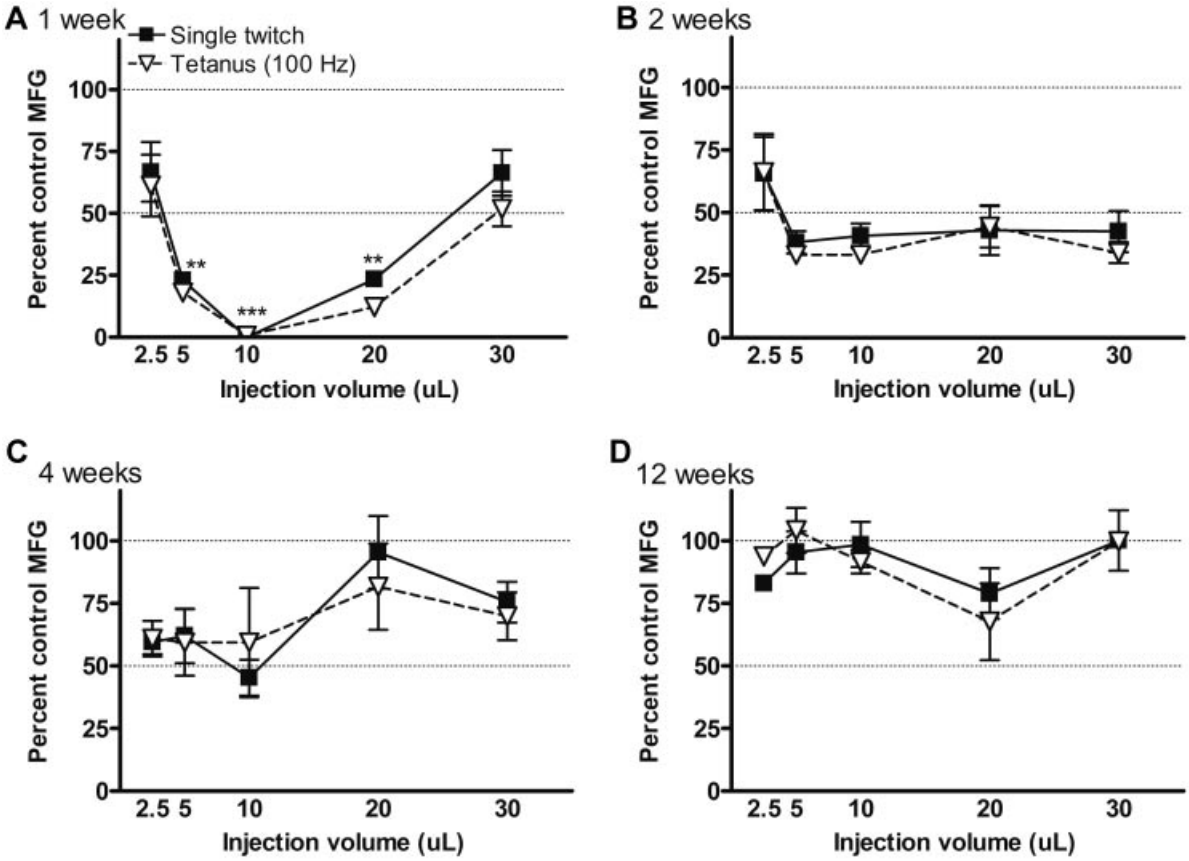

Figure 2. Effects of different injection volumes on MFG following 3 U/kg BoNTA injection. All injection volumes produced significant decrements in MFG at 1 week, but returned to near baseline by 12 weeks post-injection. (A) MFG 1 week post-injection. An injection volume of 10 μl was the most effective in reducing muscle force ($^{**}p < 0.01$ vs. 2.5 and 30 μl; $^{***}p < 0.001$ vs. 2.5 and 30 μl). (B) MFG 2 weeks post-injection. (C) MFG 4 weeks post-injection. (D) MFG 12 weeks post-injection. Control = contralateral limb.

between doses after 1 week (Table 1), but MFG in the 3 and 6 U/kg group was significantly lower than that of the 1 U/kg group MFG through week 4 for both single twitch and tetany (Fig. 1). MFG testing in combination with EMG analysis can lead to a more precise assessment of toxin effects in the animal model with greater relevance to clinical goals for muscle tone.

The volume-dependent trends (Fig. 2a) suggest that efficacy may be improved by altering injection volume. The toxin immediately diffuses to the surrounding tissues after injection, but the degree of diffusion may be affected by volume, injection site, and speed of injection in addition to the serotype and preparation of botulinum toxin.[6–8,24–26] Two previous studies noted

Table 3. Percent Control Muscle Mass following BoNTA Injection

	BoNTA U/kg Injected		
Weeks	1	3	6
1	77 ± 2	78 ± 4	63 ± 6
2	81 ± 2	79 ± 3	64 ± 3
4	74 ± 2	62 ± 2$^{\#}$	61 ± 7$^{\#}$
12	94 ± 2	91 ± 5	94 ± 8

	Injection Volume 3 U/kg BoNTA				
Weeks	2.5 μl	5 μl	10 μl	20 μl	30 μl
1	89 ± 4	70 ± 1	78 ± 4	64.6 ± 1.9	87 ± 2
2	88 ± 4	65 ± 5	79 ± 3	69.4 ± 2.8	68 ± 4
4	78 ± 3*	77 ± 3**	62 ± 2	93.2 ± 1.6***	92 ± 2***
12	93 ± 2	95 ± 5	91 ± 5	83 ± 2	94 ± 3

$^{***}p < 0.0001$, $^{**}p < 0.01$, $^{*}p < 0.05$ versus 10 μl at 4 weeks; $^{\#}p < 0.05$ versus 1 U/kg at 4 weeks.

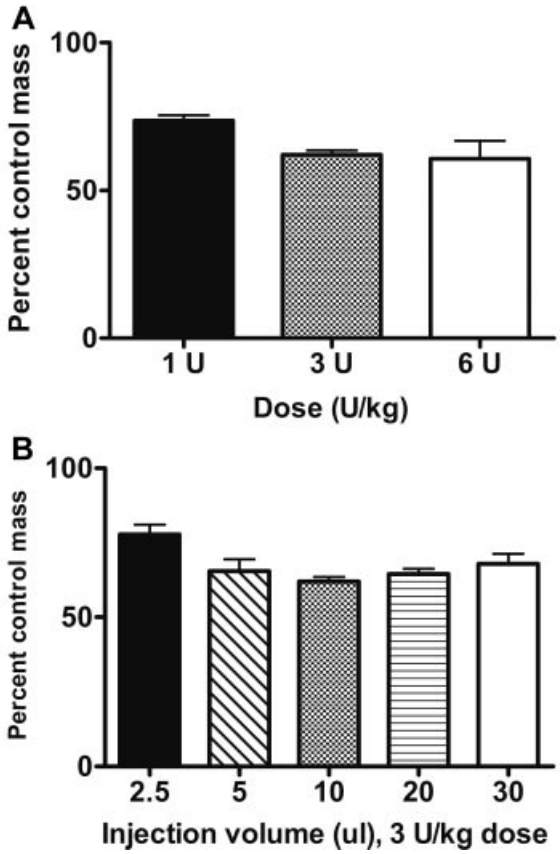

Figure 3. Peak decrements in muscle mass following BoNTA injection. (A) Dose-dependent response to BoNTA in muscle mass. Doses of 3 and 6 U/kg produced the greatest loss in mass ($^{*}p < 0.05$) at 4 weeks post-injection. (B) Volume-dependent response to 3 U/kg BoNTA on muscle mass. 10 μl produced the greatest decrement in muscle mass. ($^{**}p < 0.01$) Control = contralateral limb.

better efficacy by increasing injection volume but few injection volumes were assessed.[20,26] While the aforementioned studies do offer promising insight into the most effective volume techniques, previous studies have noted limits on the effectiveness of high dilution volumes. A fourfold dilution of botulinum toxin A (100 U/ml) did not produce statistically significant differences in the degree of paralysis in children as judged by CMAP, static muscle range and the modified Ashworth scale.[23] Another study found that a twofold dilution in intramuscular injections in humans had no significant impact on efficacy.[22] The injection volume must be large enough to effectively diffuse over the neuromuscular junctions but small enough to prevent diffusion through the fascia.[27] The studies which did not detect significant volume effects analyzed human responses and the tools for assessing physiologic responses are more limited than in animal studies. The use of an animal model allowed for more sensitive and detailed analysis of the physiology and strongly suggests significant volume-dependent effects.

Although BoNTA affects wet muscle mass in a different time course than that seen for EMG and MFG, the degree of muscle lost was still a function of the dose and volume administered (Fig. 3, Table 3). As mentioned, 3 and 6 U/kg doses produced the greatest decrement in mass (similar to EMG and MFG results), and a 10 μl injection volume is again a more effective volume for intramuscular injection. The muscle appears to atrophy rapidly with a high dose (6 U/kg) even though the degree of EMG and MFG inhibition was no different than 3 U/kg. Higher doses of BoNTA may cause a prolonged attenuation of the normal, spontaneous acetylcholine release, or prevent the development of sprouts. In the rat, chemically denervated skeletal muscle follows a time course similar to surgically denervated muscle in the changes of contractile properties, such as muscle phenotype, twitch response, and capillary density.[28–30] Muscle atrophy is accompanied by significant remodeling of the vascular bed, so if all tone is lost and the minute acetylcholine release is abolished, capillary blood flow could be rapidly reduced within a week of denervation.[30,31] It is possible that this loss of blood flow could account for the rapid muscle atrophy associated with higher doses.

The volume-dependent responses observed in this study have important clinical implications for intramuscular injections. If injection volume is too great, the degree of inhibition of a 3 U/kg dose actually approximates the inhibition accomplished with a 1 U/kg dose. This observation may indicate that the BoNTA might be better administered in terms of approximating the number of neuromuscular junctions rather than relying on body weight as an indicator for the number of units injected. This idea is supported by the fact that the doses used for mice in this study and a previous study use roughly a fifth of the dose used in the rat to accomplish a similar degree of inhibition.[19] Initial observations also suggest that the mouse gastrocnemius has approximately a fifth of the neuromuscular junctions (data unpublished) of the rat gastrocnemius.[32] Identifying the lowest dose capable of producing the desired inhibition in the smallest injection volume is critical for minimizing patient risks associated with BoNTA permeating neighboring muscles and the development of antibody resistance to BoNTA.[8,33] Antibody formation to BoNTA can result from repeated injections administered in a short time period, injections of high number of toxin units from overestimating the U/kg body weight ratio necessary to inhibit response, and the cumulative dose administered over multiple treatments.[33]

The use of the uninjected contralateral limb as the control may be considered a limitation; however, this approach was reasonable based on previous results. Control values generated in the contralateral limb were within the ranges of the previously published injected and uninjected legs MFG data in the same mouse gender and strain.[19] Since pilot study mice produced MFG in the contralateral limbs within previous control ranges, it was not deemed necessary to inject contralateral limbs with saline in this study. Injection

limbs were not randomized to side; however, injected values for the 3 U/kg dose in 10 μl were consistent with previously published values.[19] Although histology may confirm the morphometric changes in the atrophied muscle and its neuromuscular junctions, alterations in neuromuscular junction remodeling, and muscle recovery are already well documented.[1,5,12,13,15,16,19,34,35] This study explores functional outcomes after varying injection dose and volume, and recognizes that it is limited to MFG testing, EMG testing, and muscle mass.

Intramuscular injection of BoNTA affects MFG, EMG testing, and wet muscle mass in a dose- and volume-dependent manner. The present study suggests that full and partial inhibition of MFG may be accomplished by varying the dose, while varying the injection volume of a specific dose can optimize delivery. These findings have important clinical implications in the intramuscular injection of BoNTA in managing muscle tone. Previous investigation into the use of botulinum toxin for bioprotection of tendon repair is indicative of a beneficial response partial reversible chemical denervation, but others have noted that complete removal of load can be detrimental in a different muscle tendon unit, the rotator cuff.[5,36] The ability to tightly control the degree of tone inhibition may be accomplished by varying the dose and volume to maximize the delivery in a larger muscle, such as the gastrocnemius, or targeting a small muscle with a tendon injury, such as the supraspinatus or the flexor digitorum profundus. Our investigation into the dose and volume relationship for intramuscular BoNTA injections may lead to refinement of dose and volume injection techniques and subsequent improvement in the management of muscle tone.

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