Effects of Botox® and Neuronox® on Muscle Force Generation in Mice

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Received 19 September 2006; accepted 20 April 2007

Published online 28 June 2007 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jor.20450

ABSTRACT: The current study determined the dose–response relationship for inhibition of muscle force of two commercially available botulinum neurotoxin type-A (BoNTA) preparations (Botox® and Neuronox®) in a murine model and characterized the time course of recovery from the toxin-induced muscle paralysis. The effect of freezing reconstituted toxin on toxin potency was also determined. The gastrocnemius muscles in male CD-1 mice were injected with either saline or BoNTA (0.3–3.0 U/kg), and muscle force generation was examined following stimulation of the tibial nerve (single twitch and 15–200 Hz tetany). Botox and Neuronox produced nearly equivalent decrements in muscle force (30%–90%) at 4 days after toxin injection. At 28 days after injection (1 U/kg), muscle force had recovered from the effects of both toxin preparations. Maintaining reconstituted toxin at −80°C for up to 5 months did not result in significant loss of toxin activity. The results of this study suggest that Botox and Neuronox produce equivalent responses in a murine model, and, in contrast to other models, muscle recovery is rapid with doses of toxin that produce less than maximal decrements in muscle force. © 2007 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 25:1658–1664, 2007

Keywords: botulinum toxin; botulinum neurotoxin A; dose; muscle recovery; mouse

INTRODUCTION

Botulinum neurotoxin type-A (BoNTA) is a zinc-dependent endopeptidase that specifically targets the neuromuscular junction.[1–6] The active form of BoNTA is comprised of two subunits, the heavy chain and light chain, linked by a single disulfide bond.[7–9] Endocytotic uptake of BoNTA causes a light chain conformational change which results in the proteolytic cleavage of the docking protein SNAP-25.[3,9] Cleavage of SNAP-25 ultimately paralyzes the muscle by preventing the vesicular release of acetylcholine into the synaptic cleft.[3,9–11] The resulting chemodenervation halts muscle growth and results in decreased fiber size and myonuclear number within 7 days.[12]

Although BoNTA blocks the neuromuscular junction, the muscle paralysis it produces is only temporary. Recovery in humans is seen within 3–6 months and is achieved in rodents at a much more rapid rate.[2,4,6,11,13–15] Increases in nerve growth factors and evidence of myonuclear addition (through myoD) are seen in 2 days, while increased motor end-plate activity in the area of injection, indicative of axonal sprouting, is often seen in about a week.[12,16–18] Functional recovery can be achieved with the development of new nerve terminals, but full recovery may be unattainable following injections with high toxin concentrations.[6,14,15,19]

BoNTA's ability to cause partial and reversible muscle paralysis makes it a clinically useful tool for treatment of muscle overactivity and has been used to treat many disorders including achalasia, hyperhydrosis, chronic anal fissure, and spasticity.[2,4,5,11,13] BoNTA has been highly effective in treating spasticity associated with pediatric cerebral palsy since the early 1990s.[4,5,10,11] Despite its widespread use in managing muscle spasticity, controversy still exists over the appropriate dosing techniques required to achieve specific clinical goals in the reduction and management of spasticity.[4,5,11,14,20]

The present study uses a highly sensitive and reproducible model to quantify changes in muscle force generation in response to BoNTA injections. After verification of the structural integrity and biological activity of frozen reconstituted BoNTA, a dose–response curve was established and time course recovery analyzed through the measurement of muscle force generated by the mouse gastrocnemius. The intent of the study was to

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determine whether Botox® and Neuronox® exhibited similar behavior in their degree of muscle paralysis and the rate of recovery.

MATERIALS AND METHODS

Approval for the use of mice in this study was obtained from the Wake Forest University School of Medicine Animal Care and Use Committee. All portions of the protocol were performed in accordance with the guidelines detailed in the *Guide for Care and Use of Laboratory Animals* published by the National Academy of Sciences.

Male CD1 mice (20–25 g, $n = 106$) were obtained from Charles River Laboratories (Wilmington, PA). Mice were housed in a vivarium with a 12 h light/dark cycle and a controlled temperature. Rat chow and water were provided ad libitum.

Botulinum Toxin Injection

To evaluate responses to freshly reconstituted toxin, a single bottle of lyophilized botulinum neurotoxin type-A (Botox, 100 U; Allergan, Irvine, CA; or Neuronox, 100 U; Medytox, Kak-rl, Ochang-myeon, Cheongwon-gun, Chungcheongbuk-do, Korea) was reconstituted in 1.0 mL 0.9% saline. Aliquots of 50 μl were then either frozen in liquid nitrogen and stored at −80°C, or diluted in saline to the injection concentration (2.5 U/ml for the 1.0 U/kg dose) and immediately injected into the mouse gastrocnemius.

Mice were weighed, and anesthetized with isoflurane (IsoFlo, Abbott Laboratories, North Chicago, IL). Injections were performed percutaneously with the aid of a dissecting microscope (Wild-Heerbrugg M650, Switzerland) using a 25 μl Hamilton syringe equipped with a 30-gauge needle. Hind limbs were shaved, stretched and taped, then wiped with 70% ethanol to aid in visualization of the tendons of the gastrocnemius. Frozen toxin was diluted in 0.9% NaCl to 7.5, 2.5, or 0.75 U/ml, such that 0.4 μl/kg body weight injection would produce the desired dose. A dose of 3.0, 1.0, or 0.3 U/kg body weight of either Botox or Neuronox was injected into the gastrocnemius. To ensure maximal inhibition, 50% of the total dose volume was injected into each head of the muscle. The contralateral leg received an equivalent volume of saline (0.4 μl/g body weight) injected in the same fashion. Mice were ear tagged and returned to their cage.

Assessment of Muscle Force Generation

Mice were retrieved for testing for all doses of both Botox and Neuronox at 4 days following injection. Time-dependent recovery was measured for 1 U/kg doses of Botox and Neuronox at 14 and 28 days postinjection.

Mice were anesthetized using isoflurane and remained under gas anesthesia for the duration of the experiment. The sciatic nerve and gastrocnemius were isolated from surrounding tissues by an open incision. Mice then were transferred to a platform and muscle force generation was assessed according to the technique described by Ma et al.[14] with the following modifications. A bipolar parallel hook electrode (FHC, Bowdoinham, ME) connected to a stimulator (SD9, Grass) was used to stimulate the sciatic nerve. The nerve was then stimulated to elicit maximal single twitch response (0.10 V–2.0 V, 0.5 ms duration) and then the frequency of stimulation was varied (0.950 V; 15, 25, 40, 60, 100, 150, 200 Hz) to elicit tetany with 120 s rest between each tetanic stimulation. Peak force produced by tetanic stimulation occurred within 200 ms of stimulation. Stimulation voltages were verified using a digital oscilloscope (Tektronix TDS 220, Richardson, TX). The Achilles tendon was connected to a force transducer (model FT03, Grass) using stainless steel suture (Aristia Surgical, New York, NY). The force transduction signal was amplified (model 13-G4615-50, Gould, Cleveland, OH) and recorded on a calibrated recording oscillograph (RS 3800, Gould). The procedure was repeated for the contralateral (saline control) leg. Animals were euthanized at the end of the experiment.

Control animals ($n = 18$) receiving no injections into either leg were tested in accordance with the above protocol to further validate the model.

Microfluidic Analysis of Fresh and Frozen BoNTA

BoNTA samples were analyzed through a microfluidic preparation. Samples were prepared according to the protocol provided with the Protein 200Plus LabChip Kit (Agilent Technologies, Palo Alto, CA) and analyzed on the Aglient 2100 Bioanalyzer (Aglient Technologies). BoNTA was analyzed under both reducing and non-reducing conditions (reduced using β-mercaptoethanol; Sigma, St. Louis, MO). To establish the fresh toxin baseline, a vial of botulinum toxin-A (10 ng, #130A; List Biological Laboratories) was reconstituted in 400 μl 0.9% saline. Four samples were each analyzed under nonreducing and reducing conditions.

An additional vial of BoNTA (10 ng, #130A; List Biological Laboratories, Campbell, CA) was reconstituted in 60 μl 0.9% saline. Aliquots of 10 μl were then stored at one of three temperatures (4°C, −20°C, or −80°C) for 1 week prior to analysis. One aliquot stored at −80°C was subjected to an additional freeze/thaw cycle prior to analysis for a total of two freeze/thaw cycles. These samples were then subjected to microfluidic analysis using the Agilent 2100 Bioanalyzer.

Statistical Analysis

Statistical analysis was performed using Prism 4.0 (GraphPad). Linear and nonlinear regressions were generated using data from uninjected control animals to determine the effect of body weight on muscle force generation. One and two-way ANOVAs were used to analyze drug, dose, and time effects. When significant main effects were found, 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 ±SEM unless noted otherwise in the figure legend.

RESULTS

In control saline-injected animals, there was a linear relationship between body weight and muscle force generation for body weights up to approximately 35 g. With body weights greater than 35 g, the relationship deteriorated and formed a plateau for maximal force generated (Fig. 1). In animals receiving toxin, force generated in the contralateral saline-injected muscle did not differ significantly from the values obtained from the uninjected control animals ($p = 0.82$), indicating that the injected toxin did not affect the contralateral leg. For clarity, all dose and time-dependent responses are reported in terms of percent contralateral leg. Fresh and frozen toxin studies are reported as absolute force because bodyweights were very similar and fell within the linear range.

Fresh versus Frozen Toxin: Microfluidic Analysis

Under nonreducing conditions, a single band was easily identifiable at ~144 kDa (Fig. 2), which is congruent with the baseline achieved with fresh toxin (data not shown) and published values for BoNTA obtained during SDS-PAGE experiments.[7,8,21] In addition to a small amount of unnicked toxin at 144 kDa, two bands were produced under reducing conditions at approximately 93 kDa and 51 kDa that respectively correspond to the heavy and light chains of BoNTA.[7,8,20] Nonreducing conditions did not show any evidence of BoNTA's deterioration into its subunits. The structural integrity of the toxin appears to be maintained following reconstitution when stored at −80°C, −20°C, and 4°C; furthermore, the toxin appears stable through two repeated freeze/thaw cycles.

When compared to freshly prepared and injected BoNTA, previously frozen reconstituted aliquots did not exhibit a reduction in potency in inhibition of muscle force generation in the study mice. As evidenced in Figure 3, both the fresh and frozen injections of the neurotoxin (1U/kg, either Botox or Neuronox) reduced the muscle force generation to approximately 30% of the control. The force generated by the control legs did not differ significantly between the fresh or frozen toxin treatments ($p = 0.82$). The toxin-injected legs did not exhibit a statistically significant difference from one another ($p = 0.71$ and $p = 0.90$ for BoNTA and Neuronox, respectively), but toxin injections caused a significant decrease in the force generation compared to contralateral legs ($p < 0.01$).

Dose-Dependent Response

Doses of 0.3 U/kg, 1 U/kg, and 3 U/kg were administered to generate a dose–response curve for Botox- and Neuronox-treated animals. While many frequencies between 15 and 200 Hz were tested, for clarity, only single twitch, 60 Hz, 100 Hz, and 150 Hz are shown in Figures 4

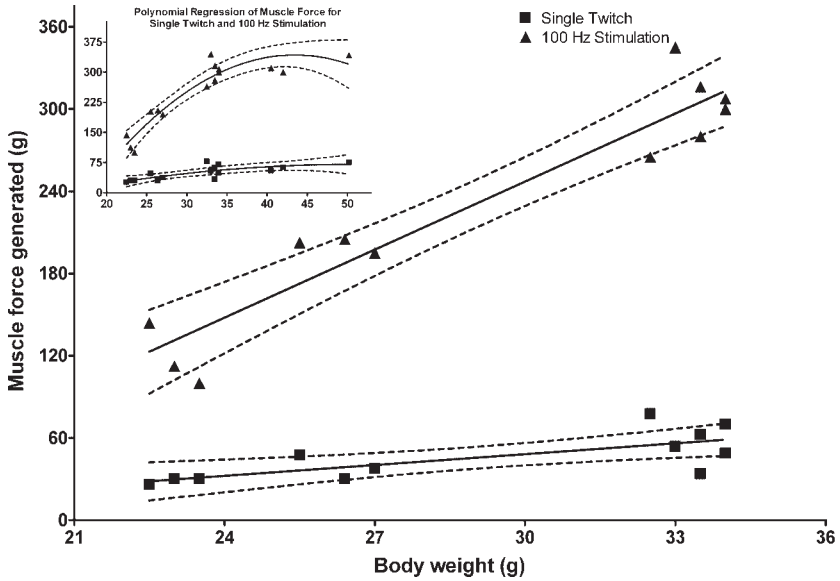

Figure 1. Effects of body weight on muscle force generated by uninjected control mice. A linear relationship exists between body weight and muscle force generated ($R^2 = 0.90, p < 0.05$). The linear relationship deteriorates after approximately 35 g when it appears mice increase body fat percentage rather than muscle mass (inset). Dotted lines represent the 95% confidence interval.

 DOI 10.1002/jor

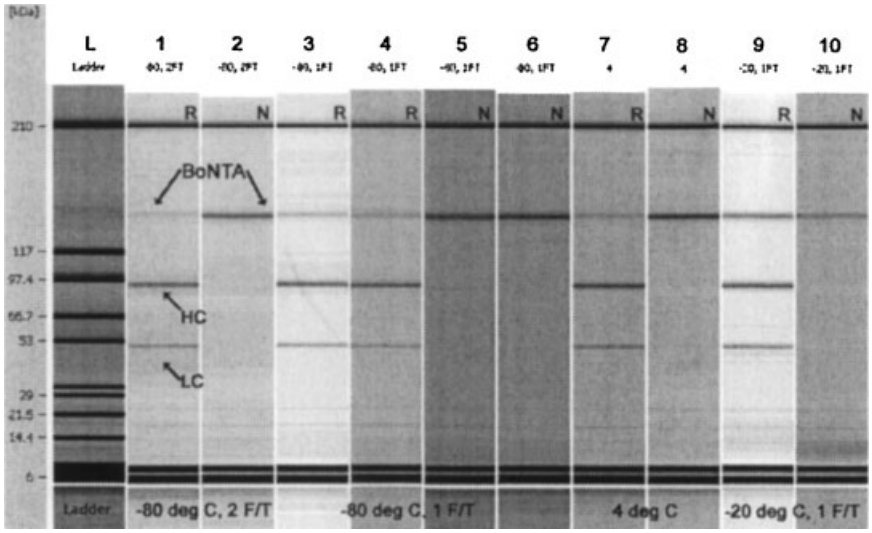

Figure 2. Gel representation of purified BoNTA following microfluidic electrophoretic analysis. Microfluidic analysis conducted under conditions representative of a 6%–15% gradient SDS-PAGE gel. Lanes are labeled above and below the gel [L, Ladder; (1,2), −80°C, two f/t cycles; (3–6), −80°C, one f/t cycle; (7,8), 4°C; (9,10), −20°C, one f/t cycle), and reducing (R) and nonreducing (N) conditions are shown at the top of the lane. Bands at 6, 9, and 210 kDa are system peaks. Nonreduced BoNTA is visible under both reducing and nonreducing conditions at ~144 kDa. Reducing conditions produce two additional bands at ~93 and ~51 kDa that respectively correspond to the heavy and light chains of the active toxin. These bands are absent under nonreducing conditions. (HC, BoNTA heavy chain; LC, BoNTA light chain; f/t, freeze/thaw.)

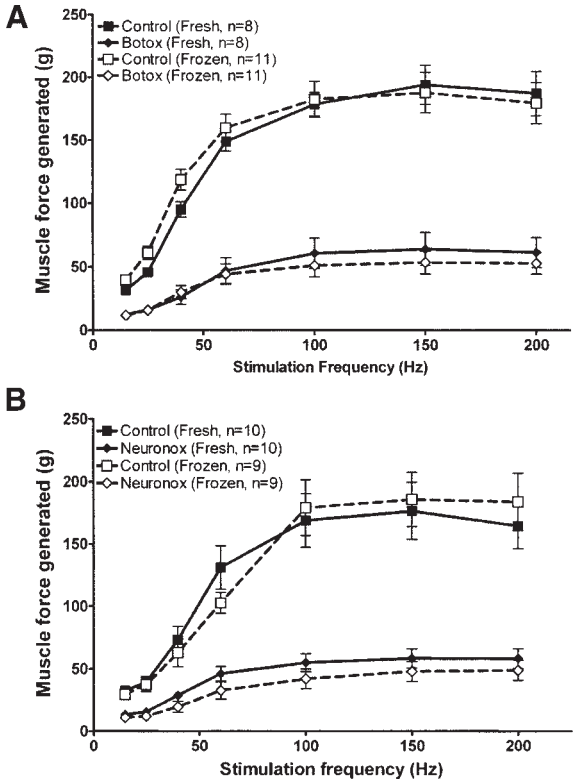

Figure 3. Muscle force generated after 1 U/kg fresh or frozen BoNTA (Botox or Neuronox). (A) Contralateral muscle force versus injected legs of both fresh (—) and frozen (- -) reconstituted Botox. (B) Contralateral muscle force versus injected legs of both fresh (—) and frozen (- -) reconstituted Neuronox.

and 5. A dose of 3 U/kg Botox or Neuronox produced near total inhibition, as the injected leg was able to produce only 8% and 5% of the contralateral muscle force generation, respectively (Fig. 4). Both 1 U/kg and 0.3 U/kg produced a partial inhibition of force generation. A 1 U/kg dose of Botox resulted in a force generation 30% of control force; Neuronox-treated mice responded almost identically with 30% of control force (Fig. 4). Slight inhibition was accomplished using 0.3 U/kg, which reduced force to 48% and 64% of controls with Botox and Neuronox, respectively (Fig. 4).

Time Course Recovery

Mouse legs injected with 1 U/kg Botox or Neuronox were able to generate only 30% of the force produced by the contralateral leg across both single twitch and tetany (Figs. 4 and 5). At 14 days postinjection, recovery for Botox and Neuronox reached respective values of 71% and 78% of the contralateral muscle force (Fig. 5). By 28 days after injection, the injected leg generated forces 92% of the contralateral leg when injected with Botox and 124% when treated with Neuronox (Fig. 5). An unpaired *t*-test demonstrated that by 28 days postinjection, the absolute force values between control and injected legs were not significantly different from one another (range of

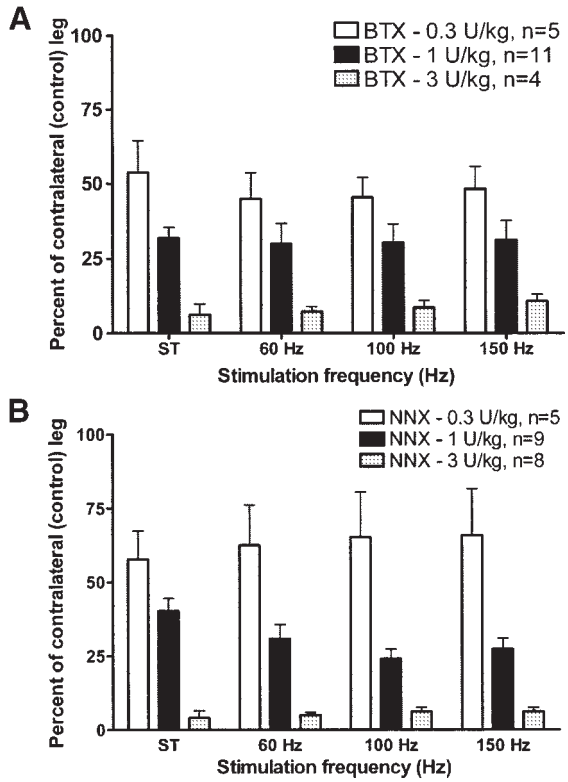

Figure 4. Dose response in muscle force generated after BoNTA injection. (A) Botox-injected mice showed near complete inhibition by 3 U/kg body weight dose with mild inhibition achieved at a 0.3 U/kg dose. (B) Neuronox-treated mice responded similarly to Botox-treated mice. All means are expressed as percent of contralateral leg. (ST, single twitch; BTX, Botox; NNX, Neuronox.)

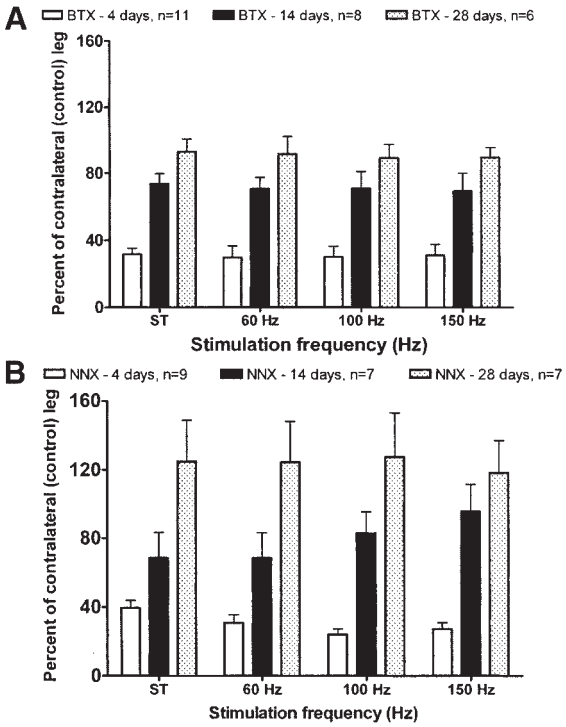

Figure 5. Muscle force recovery reached by 28 days after 1 U/kg BoNTA injection. (A) Botox-injected mice tested at 4, 14, and 28 days reach full recovery. (B) Neuronox-injected mice tested at 4, 14, and 28 days postinjection reach full recovery. (ST, single twitch; BTX, Botox; NNX, Neuronox.)

$p = 0.09–0.68$) regardless of drug. A two-way ANOVA demonstrated that all recovery time points were significantly different from one another ($p < 0.001$) but that there was no significant difference between the two toxins in the rate ($p = 0.99$) or degree ($p = 0.98$) of recovery.

DISCUSSION

The muscle force generation model provides a reproducible and accurate assessment of the degree of functional inhibition produced by intramuscular injection of two toxin preparations. A linear relationship existed between body weight and muscle force generation during the rapid growth period where more muscle was added than fat (Fig. 1). At approximately 35 g, the relationship deteriorated, presumably as body fat percentage increased. The muscle force generation of the contralateral legs of injected mice attained the predicted values based on the uninjected control values. Because the contralateral legs matched the uninjected controls in force generation, there was no evidence of systemic effects from toxin injections.

Mice were tested 4 days after injection to ensure maximal inhibition by BoNTA prior to the onset of significant muscle atrophy. In rats, maximal effects occurred at approximately 3 days, but significant atrophy did not occur until 1 week after injection.[14] Previous studies found the toxin began to act within hours of injection,[18,22] but growth factor increases did not appear until at least 2 days later, and sprouting did not occur until at least 2–3 days after injection in mice, and was more delayed in rabbits and humans.[16–18,23] A 1 U/kg dose was optimal to assess the efficacy of BoNTA in vivo, because it produced substantial but not total inhibition with low variability, so small losses in efficacy could be resolved. Following a comparison of surgically opened injections (method of Ma et al.[14]) versus closed-skin injections, it was established that the closed skin protocol did not impact the localization, success, or efficacy of the injected toxin (data not shown). Closed-skin injections also provided a cleaner surgical preparation for muscle force generation testing. As shown in

Figure 3, no loss in efficacy was seen after storage of the toxin aliquots at −80°C and a single freeze/thaw cycle. The contralateral controls did not show any signs of systemic toxicity indicating that the toxin effectively remained localized at the site of injection.

To confirm in vivo efficacy following a freeze/thaw cycle, the contents of a BoNTA injection were analyzed using microfluidic analysis (Fig. 2). A single band under nonreducing conditions indicated that no degradation occurred following storage at −80°C, −20°C, and 4°C after either one or two freeze/thaw cycles. Should the toxin be broken at the disulfide bond, bands should appear in the ranges of the subunits found under reducing conditions (Fig. 2, markers HC and LC). No additional bands were found that provide evidence of complex formation from damaged protein fragments. These observations are consistent with previously published electrophoretic characterizations of BoNTA.[7,8,21]

Once it was established that freezing the toxin did not impair its function, all studies were conducted using previously frozen toxin. The ability to store reconstituted toxin frozen for short periods of time with little loss in efficacy reduced the cost of animal studies with commercially produced toxin preparations. It was previously reported that some serotypes of botulinum toxin have been shown to retain their toxicity following a single freeze/thaw process, but damage is caused that affects toxin properties such as heat inactivation[24]; the present study did not address these other properties, but found no externally apparent systemic effects from injection with the frozen toxin.

Gartlan and Hoffman[25] found that reconstituted BoNTA lost up to 70% of its potency in an intraperitoneal injection LD50 assay following 2 weeks of frozen storage; however, a study to quantify decreases in effectiveness through muscle force analysis in mice had not been performed. Our results showing sustained efficacy with freezing, also parallel the findings of a previous human study where the potency of frozen and refrigerated BoNTA was assessed by measuring the decline in the human extensor digitorum brevis M-wave amplitude.[26] Sloop et al. found that this measurement of muscle paralysis was much more sensitive than the mouse LD-50 tests of toxin potency, and they too determined that there is no significant loss in efficacy following freezing or refrigerating the toxin for short periods of time.[26]

A dose-dependent curve was constructed with doses that produced full and partial inhibition of gastrocnemius function. A dose of 1 U/kg is on the low side of the clinical range[4,5,10,11,20] and much lower than published doses administered in the rat gastrocnemius[6,14,21]; however, doses greater than 1 U/kg produced complete inhibition of function of the mouse gastrocnemius. A high degree of muscle sensitivity to very small doses of BoNTA in the mouse gastrocnemius could be related to the presence a smaller number of neuromuscular junctions per kilogram body weight and may also be attributed to significant paralysis achieved through cleavage of only a small percentage of SNAP-25.[9] Our model verifies that significant changes in function may occur at lower dose levels. Future studies may be performed to identify the most effective doses to achieve specific functional goals.

The low dose used for recovery analysis for toxin-injected animals demonstrated a clear recovery pattern which reached 100% recovery within 28 days of the injections (Fig. 5). The rapid rate of recovery suggests that partial inhibition is easily overcome in the murine model and differs from the rate of recovery in the rat gastrocnemius.[19] The time and degree of recovery did not differ significantly between Botox and Neuronox. Congruencies between the degree of inhibition and both the rate and degree of recovery indicate that Neuronox and Botox may be substituted in a 1:1 ratio in dosing for mouse studies.

The lower dosing may be an effective tool in the clinical administration of BoTNA. If partial paralysis is sought with the intended goal of full recovery, administering a concentrated dose could not only result in the total loss of tone but may also limit muscle functional recovery. The present study offers some insight into the dosing technique for BoNTA injections and mouse muscle force and establishes the ability to substitute Botox and Neuronox unit for unit in mice. This model is a useful tool for measuring the impact of drugs on in vivo neuromuscular function. Additional studies on the dose to muscle force relationship may yield insight into more effective dosing techniques to accomplish clinical goals in managing spasticity and muscle function.

ACKNOWLEDGMENTS

This study was supported by Revance, Inc., the Institute for Regenerative Medicine at the Wake Forest University School of Medicine, and the MusculoSkeletal Research Center at the Wake Forest University School of Medicine, Winston-Salem, NC. Special thanks to Dr. Delrae Eckman for his editorial comments. Portions of this manuscript were presented at the 2006 Meeting of Experimental Biology.

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