

Short communication

Frequency and length-dependent effects of Botulinum toxin-induced muscle weakness

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Abstract

While the pathogenesis of Botulinum toxin type A (BTX-A)-induced muscle weakness has been systematically researched, little is known about the effects of motor fibre paralysis on the mechanical properties of skeletal muscle. Here, the long-term effect of BTX-A injection on the force–length and force–frequency properties of rabbit knee extensors is investigated. BTX-A-induced muscle weakness was greater at short compared to long muscle lengths and at low compared to high stimulation frequencies four weeks following intervention. Therefore, we conclude that the paralyzing effects of BTX-A are not uniform, and must be considered in biomechanical models of musculoskeletal rehabilitation in which BTX-A is used therapeutically, as for example in patients with cerebral palsy. Although the present results could be explained through a variety of mechanisms, this study does not allow for drawing firm conclusions about the length and frequency-dependent effects of BTX-A.

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1. Introduction

Botulinum toxin type A (BTX-A) is one of seven neuromuscular blocking agents produced by the bacterium *Clostridium botulinum*. It has been used therapeutically in the treatment of numerous medical disorders for many years (Brin, 1997). As experience with this drug has increased, so too has our understanding of its mechanism of action. BTX-A has a relative specificity for motor nerve terminals where it inhibits spontaneous and evoked neurotransmitter release (Habermann and Dreyer, 1986). More specifically, after binding to the axon terminal of the alpha-motoneuron, the toxin is internalized by means of a receptor-mediated endocytosis. Once inside the cell, BTX-A prevents neurotransmitter release by inhibiting the fusion of acetylcholine (ACh)-containing vesicle membranes with the cytoplasmic membrane of the motor nerve ending (Brin, 1997).

Without ACh release, muscle fibres cannot be physiologically activated.

While the pathogenesis of BTX-A induced muscle weakness has been elucidated, our knowledge of the effects that motor fibre paralysis has on muscle mechanics is incomplete. Currently, it is known that the force-development properties of normal muscle are profoundly dependent on the frequency and length at which the muscle is stimulated (e.g. McComas, 1996). With the goal of investigating the implications that muscle fibre weakness has on mammalian muscle mechanics, this study examined the degree of BTX-A induced weakness present at different muscle lengths and stimulation frequencies in the New Zealand white rabbit.

2. Materials and methods

2.1. Experimental design

Skeletally mature, one-year-old New Zealand white rabbits (4.1–6.5 kg, Riemens, St. Agatha, Ont., Canada)

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were obtained and studied with the approval of the Animal Care Committee of the University of Calgary. Animals were housed locally in accordance with Canadian Council on Animal Care Guidelines. All animals were allowed normal activities in a cage $65 \times 45 \times 30 \text{ cm}^3$. The rabbits received a standard diet and water ad libitum.

Ten animals were divided into two study groups for maximal isometric knee extensor torque analysis at varying knee angles and stimulation frequencies. Group one consisted of five control rabbits. Group two consisted of five rabbits which, four weeks prior to testing, received a one-time intramuscular injection of BTX-A unilaterally into the quadriceps musculature of the hindlimb. The maximal isometric knee extensor torques were compared between hindlimbs using implantable femoral nerve cuff stimulators. Following testing, the rabbits were sacrificed by an overdose injection of Euthanyl (MTC Pharmaceuticals; Cambridge, Ont., Canada) into the lateral ear vein.

2.2. *Botulinum type A toxin (BTX-A): injection protocol*

Each 100 unit vial of vacuum-dried *Clostridium botulinum* type-A neurotoxin complex (BOTOX, Allergan, Inc., Toronto, Ont., Canada) was reconstituted with 0.9% sodium chloride (without preservative) to a concentration of 20 units/ml. All rabbits in Group two received a one-time intramuscular BTX-A injection at a total dose of 3.5 units/kg. One unit of the drug corresponds to the calculated median intraperitoneal lethal dose (LD50) in mice. The injection was randomized to either the right or left quadriceps musculature of the rabbit hindlimb. Prior to injection, rabbits were sedated with a 0.18 ml subcutaneous injection of Atravet (10 mg/ml) (Acepromazine; Ayerst Laboratories, Montreal, Que., Canada). A general anaesthetic was induced using a 2% halothane/oxygen mixture. The anterior compartment of the thigh, containing the quadriceps musculature, was isolated by manual palpation. BTX-A was injected intramuscularly into the quadriceps with a 30-gauge needle, once the needle had been felt to penetrate the overlying fascia. Although the size of the denervation field induced by BTX-A is largely dose and volume dependent (Borodic et al., 1994; Shaari and Sanders, 1993), the injection protocol consisted of visually dividing the quadriceps into superior and inferior halves. Each half was further subdivided into medial, central, and lateral sections. One sixth of the total BTX-A dose was injected into each section to increase toxin diffusion. A sham injection was performed into the contralateral quadriceps muscle using the identical procedure and an equal volume of 5% dextrose/0.9% sodium chloride solution (D5W). Group one served as control rabbits and did not receive any injections.

2.3. *Outcome measures: isometric knee extensor torque*

Isometric knee extensor weakness, the primary outcome measure for this study, was calculated as the difference in maximum isometric torque between the experimental and contralateral hindlimb for each test condition in the experimental animals. For the control animals, isometric knee extensor difference was calculated as the difference in maximum isometric torque between the left and right hindlimb for each test condition. Knee extensor torques were obtained by electrical stimulation of the knee extensor muscles through a femoral nerve cuff electrode that was implanted prior to data recording. In the ideal case, knee extensor torques should have also been measured in the experimental group prior to BTX-A injection. However, implanting the nerve cuff chronically for four weeks may have influenced the primary outcome results. Therefore, we had to assume that the results of the control group represented the knee extensor torque status of the experimental animals prior to BTX-A injection, a feasible, but unproven assumption. The animals were anaesthetized as described previously. For each hindlimb, a 5 cm inguinal incision was made and dissection of the femoral nerve performed. The nerve was consistently identified at a point distal to the inguinal ligament and lateral to the femoral artery and vein. The inguinal ligament was released in order to adequately trace the femoral nerve proximally, ensuring that the stimulation cuff was applied at a position prior to any nerve branching. The animal was secured in a stereotactic frame with multiple pointed metal rods. This fixation ensured that consistent hip angles were achieved throughout testing, and allowed only tibial extension in the sagittal plane at the knee.

Following fixation, the tibia was restrained with an instrumented bar placed just proximal to the bimalleolar axis. A custom-built force sensor (sensitivity = 0.1 N; linearity in calibration: $r^2 > 0.99$), using a half-bridge strain gauge configuration and Vishay 2100 amplifier (Raleigh, NC, USA), was used to measure maximum isometric knee extensor torque. All information was recorded using Windaq data collection and playback software (Dataq Instruments, Akron, OH, USA) and a DI-400, 12 bit, $\pm 10 \text{ V}$ data collection card.

Maximum isometric knee extensor torque was recorded from each hindlimb at multiple knee angles and stimulation frequencies. All measurements were obtained by one independent observer who was blinded to the treatment group. Determination of knee angle was made with a hand-held goniometer positioned using consistent anatomical landmarks (greater trochanter of the femur; lateral joint line of the knee; lateral malleolus of the tibia). Torque measurements were recorded at three knee angles: (1) small ($75\text{--}85^\circ$); (2) medium

(95–105°) and (3) large (115–125°). Full knee extension was defined as 180°.

For each knee angle, maximum isometric knee extensor torque was measured over a range of stimulation frequencies. Each stimulation condition was tested once at each knee angle in the order single twitch, 10, 50, 100 and 200 Hz. The 100 Hz and twitch stimulation were then repeated for reliability. No systematic differences were found for repeat measurements, and all measurements (including the repeat tests at 100 Hz and the single twitch) were used for analysis. Stimulation was given through a dual output Grass S8800 stimulator (Astro-Med Inc., Longueuil, Quebec, Canada) at a voltage three times higher than the alpha-motoneuron threshold, to ensure activation of all motor units (Herzog and Leonard, 1997, 2002). Stimulation duration was 500 ms, with a one minute rest period between stimulations to avoid fatigue. Individual pulse durations were 0.1 ms with a rectangular square wave.

3. Results

Muscle weakness increased from small to large knee angles in a systematic way (Table 1). That means that the decrease in muscle strength associated with BTX-A injections (i.e., weakness) was greatest at short and smallest at the long muscle length. This increase averaged 9% \pm 2% (mean \pm SD) and was statistically significant on analysis with a one-way ANOVA followed by Bonferroni's multiple comparison adjustment ($p < 0.01$). Identical analysis performed on the control rabbits (Table 1) revealed no differences in left and right knee torques for the three knee angles tested.

Muscle weakness in the experimental rabbits between BTX-A injected and contralateral hind limbs increased

Table 1
Muscle weakness for experimental (four-weeks post BTX-A injection) and control rabbits

Group	Knee angle	Muscle weakness [%](\pm 1SD)
Experimental	75°–85°	74% (12%)
Experimental	95°–105°	79% (12%)
Experimental	115°–125°	83% (13%)
Control	75°–85°	1% (13%)
Control	95°–105°	4% (16%)
Control	115°–125°	–6% (37%)

Percent weakness reflects the relative decreases in torque between BTX-A injected and contralateral hindlimbs (experimental) and the differences between left and right knee torques (control), averaged over the range of stimulation frequencies tested (single twitch, 10, 50, 100, 200 Hz). Means and standard deviations are shown. A significantly greater torque deficit (greater weakness) exists at large versus small knee angles ($p < 0.01$) in the experimental animals, but not the control group.

Table 2
Muscle weakness for experimental (four-week post BTX-A injection) and control rabbits, averaged over the range of knee angles tested

Group	Frequency	Muscle weakness [%](\pm 1SD)
Experimental	Single twitch	90% (\pm 5%)
Experimental	10 Hz	89% (\pm 5%)
Experimental	50 Hz	77% (\pm 6%)
Experimental	100 Hz	67% (\pm 9%)
Experimental	200 Hz	66% (\pm 9%)
Control	Single twitch	–8% (\pm 34%)
Control	10 Hz	7% (\pm 17%)
Control	50 Hz	4% (\pm 13%)
Control	100 Hz	4% (\pm 13%)
Control	200 Hz	5% (\pm 13%)

Percent weakness reflects the relative decreases in torque between BTX-A injected and contralateral hindlimbs (experimental) and the differences between left and right knee torques (control). Means and standard deviations are shown. The increase in muscle weakness with decreasing stimulation frequencies was statistically significant ($p < 0.01$), and comparisons between any two stimulation frequencies were all significant, except for 10 Hz vs. single twitch, and 100 vs. 200 Hz, in the experimental group. There was no difference in torques between left and right knee for any stimulation frequency in the control rabbits.

from high to low frequencies of muscle stimulation (Table 2). That means, muscle weakness associated with BTX-A injections was greatest at low and smallest at high frequencies of stimulation. A one-way ANOVA followed by Bonferroni's multiple comparison adjustment showed significant differences in muscle weakness ($p < 0.01$) in all cases except for the single twitch vs. 10 Hz, and the 100 vs. 200 Hz comparisons. The maximum increase in isometric knee extensor torque deficit was 24% \pm 7% (mean \pm SD, $p < 0.001$), found when comparing 200 Hz to single twitch stimulation. No such differences in isometric knee extensor torques were found in the control rabbits of Group one (Table 2).

4. Discussion

The results of this study suggest that both decreased frequency of stimulation, and shorter muscle lengths increase BTX-A induced muscle weakness. The magnitude of these effects would appear substantial, as they were evident despite the relatively small sample sizes. A frequency dependence of BTX-A induced muscle weakness has been reported previously by Dimitrova et al. (2002) in an acute testing protocol. They examined the acute effects of BTX-A exposure, in-vivo, on the contractile properties of the lateral rectus muscle of the eye in cats. Nerve stimulation at a frequency of 220 Hz within 7 h of BTX-A injection produced approximately 30% of the normal control tension in the lateral rectus muscle. In contrast, 50 Hz stimulation

only produced 10% of the control tension. This 20% difference in muscle weakness found at the low versus the high frequency is greater by a factor of two than the muscle weakness found in this study, for comparable differences in stimulation frequencies (11% for 50 vs. 200 Hz, Table 2). The acuity of Dimitrova's observation suggests that the etiology of this effect is secondary to the denervating effect of BTX-A, as any disuse atrophy present in our long-term BTX-A study would not have had time to develop. These authors also noted minimal alteration in the speed-related contractile properties of BTX-A exposed muscle implying that, at least acutely, all muscle fibre types were affected equally. Any preferential effects of BTX-A on selective muscle fibre types would have been expected to cause a change in contraction time or fusion frequency. Finally, a direct comparison of results across studies should account for differences in relative muscle length, which are not known for the two studies. Therefore, part of the difference in muscle weakness observed by Dimitrova et al. (2002) and by us might be explained by a difference in muscle length at which results were obtained.

Dimitrova et al. (2002) did not speculate on the pathophysiology behind this frequency dependent effect, and did not attempt to assess the long-term effects of BTX-A. However, BTX-A could conceivably influence the force production pathway of skeletal muscle at any point from signal initiation in the motor cortex, to the power stroke of the myosin cross-bridge. BTX-A is known to undergo retrograde transport from the muscle to the central nervous system (CNS), where it is capable of impairing the release of multiple neurotransmitters (Bigalke et al., 1985; Wellhoner, 1989). However, the evidence for this occurring in the doses used in this study is not fully substantiated in the literature. The frequency of motoneuron firing patterns has also been shown to be altered by BTX-A injection in the adult cat (Moreno-Lopez et al., 1994). These motoneuron effects may be centrally mediated, or may be a consequence of target disconnection of the nerve from the muscle fibre, because motor nerve trunks appear unaffected by BTX-A exposure (Burgen et al., 1949). Both the amplitude of action potentials and the conduction velocity of various nerves in the cat remain unchanged following BTX-A exposure at lethal doses for up to five hours (Brooks, 1954). While central effects from BTX-A injection have been reported, their significance remains uncertain.

Given that this investigation used a nerve cuff stimulation protocol, central influences are likely not the source of the frequency and length-dependent changes found in this study. The excitation-contraction coupling, another potential source for toxin induced alterations in muscle mechanics, also appears unaffected by BTX-A injection. Toxin exposed skeletal muscle remains responsive to direct electrical stimulation in the

face of complete muscle paralysis with indirect nerve stimulation (Burgen et al., 1949). Given these observations, one can speculate that BTX-A induces its length and frequency dependent effects on force output at the site of the neuromuscular junction.

Although BTX-A inhibits neurotransmitter release at the neuromuscular junction (Brin, 1997), the torque output measured in this study from BTX-A injected knee extensors necessitates that some ACh was released from motor nerve endings in response to femoral nerve stimulation. There are three possible scenarios in which this ACh release could have occurred. The first one being that ACh was released by functioning motor nerve endings that, secondary to the injection protocol, were not exposed to BTX-A. A study by Burgen et al. (1949) supports this scenario, as they reported normal tetanic responses and summation curves in BTX-A exposed muscle. The authors suggested that their partially poisoned rat diaphragm preparations contained some motor units that behaved normally in all ways, and some motor units in which transmission was not restored by conditioning stimuli.

The second scenario for the persistence of knee extensor torque post BTX-A injection could have been the result of muscle fibre contraction triggered by motor nerve endings that were only partially inhibited by BTX-A exposure, but not enough to eliminate all ACh release. Finally, the third scenario involves ACh release through the formation of nerve-terminal sprouts. de Paiva et al. (1999) demonstrated that a single BTX-A injection into the mouse sternomastoid muscle resulted in collateral sprouting of nerve terminals. They found that after 4 weeks, when nerve stimulation again elicited muscle contraction, only the newly formed nerve-terminal sprouts elicited the muscle contraction through ACh release, but not the parent terminals. While the explanation for this ongoing torque output may rest in a combination of these scenarios, a definitive explanation for the frequency and length dependent effects of BTX-A induced muscle weakness is currently unknown.

5. Conclusion

In summary, we have demonstrated here for the first time that BTX-A induced muscle weakness in the long-term is frequency and muscle length dependent. While there appears to be a growing consensus on the site and mechanism of action of BTX-A, our knowledge as to the functional effects of this paralysis or paresis on muscle mechanics remains limited. Continued investigation into this study's findings will likely contribute to our evolving knowledge of neurotransmitter release and muscle mechanics, physiology, and function.

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