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Do skeletal muscle properties recover following repeat onabotulinum toxin A injections?

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ABSTRACT

Onabotulinum toxin A (BTX-A) is a frequently used treatment modality to relax spastic muscles by preventing acetylcholine release at the motor nerve endings. Although considered safe, previous studies have shown that BTX-A injections cause muscle atrophy and deterioration in target and non-target muscles. Ideally, muscles should fully recover following BTX-A treatments, so that muscle strength and performance are not affected in the long-term. However, systematic, long-term data on the recovery of muscles exposed to BTX-A treatments are not available, thus practice guidelines on the frequency and duration of BTX-A injections, and associated recovery protocols, are based on clinical experience with little evidence-based information. Therefore, the purpose of this study was to investigate muscle recovery following a six months, monthly BTX-A injection (3.5 U/kg) protocol. Twenty seven skeletally mature NZW rabbits were divided into 5 groups: Control ($n=5$), zero month recovery – BTX-A+0 M ($n=5$), one month recovery – BTX-A+1 M ($n=5$), three months recovery – BTX-A+3 M ($n=5$), and six months recovery – BTX-A+6 M ($n=7$). Knee extensor strength, muscle mass and percent contractile material in injected and contralateral non-injected muscles was measured at each point of recovery. Strength and muscle mass were partially and completely recovered in injected and contralateral non-injected muscles for BTX-A+6 M group animals, respectively. The percent of contractile material partially recovered in the injected, but did not recover in the contralateral non-injected muscles. We conclude from these results that neither target nor non-target muscles fully recover within six months of a BTX-A treatment protocol and that clinical studies on muscle recovery should be pursued.

1. Introduction

Onabotulinum toxin A (BTX-A) is a treatment modality for a variety of neuromuscular disorders with the primary aim to relax spastic muscles that occur, for example, in children with cerebral palsy. Spastic cerebral palsy is associated with abnormalities in muscle tone and overactive stretch reflexes, resulting in muscle contractures and bony deformities. Patients with cerebral palsy are significantly weaker compared to typically developing children, which compromise their daily life activities (Barrett and Lichtwark, 2010; Brashear et al., 2002). Furthermore, spastic muscles in cerebral palsy have been found to have shorter fascicles but longer sarcomeres, indicating a reduced sarcomere number compared to typically developing people, Lieber and Friden (2002), Mohagheghi et al. (2007), Moreau et al. (2009) which contributes to increased passive forces and decreased strength in spastic patients (Barber et al., 2011; Wiley and Damiano, 1998).

Once injected into spastic muscles, BTX-A prevents acetylcholine release at the motor nerve endings, thereby inducing a dose-dependent muscle paralysis (Childers et al., 2004). BTX-A injections produce a reduced muscle tone, allowing muscles to stretch, thus increasing joint range of motion and functionality in patients. These outcomes will transiently increase a patient's independence and help delay the need for assistive devices and may postpone invasive surgical interventions (Berweck and Heinen, 2004; Hesse et al., 2000; Koman et al., 2001). Recently, BTX-A treatments have been combined with muscle stretching, casting, or electrical stimulation and such combined interventions were associated with better functional outcomes in spastic patients (Frasson et al., 2005; Hesse et al., 1995; Kang et al., 2007; Minamoto et al., 2007; Williams et al., 2013).

Although considered safe, and approved by the US Food and Drug Administration, BTX-A injections can cause severe adverse effects in injected and non-target muscles (Borodic et al., 1994; Frasson et al., 2011; Shaari and Sanders, 1993; Yaraskavitch et al., 2008). Schroeder et al. (2009) reported significant muscle atrophy of healthy human gastrocnemius one year following a single BTX-A injection, suggesting that the toxin might have long lasting

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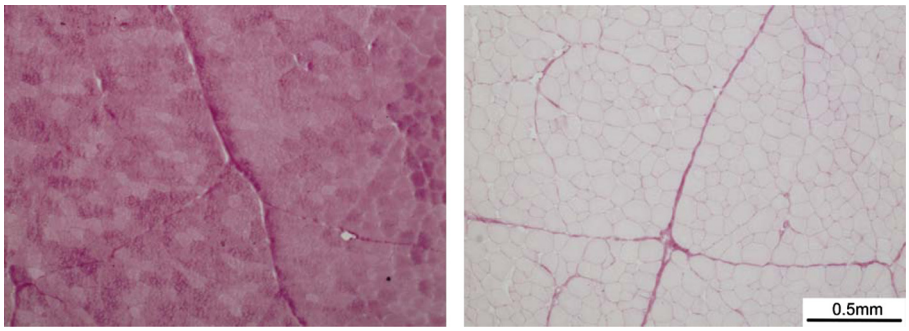

Fig. 1. Quadriceps musculature stained for glycogen with PAS following the glycogen depletion protocol. The glycogen depletion protocol consisted of fifteen minutes of continuous quadriceps muscle stimulation through the femoral nerve at a frequency of 20 Hz. It has been demonstrated that this stimulation protocol depletes all glycogen in a normally innervated muscle (Shaari et al., 1991), therefore all remnant glycogen observed following this protocol is likely associated with the blocking of activation of selected fibres through BTX-A. Left: Control muscle not exposed to the glycogen depletion protocol shows an even distribution of glycogen (red stained cells) throughout the entire cross-sectional area. Right: muscle exposed to the glycogen depletion protocol is completely devoid of glycogen. Scale bar: 0.5 mm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

adverse effects. Furthermore, repeat injections of BTX-A are performed with at least a 3 month interval, with some studies showing sustained effects of the toxin up to one year, thereby prolonging the period of reduced muscle spasticity (Brin, 1997; Graham et al., 2000; Molenaers et al., 2010). However, the detailed recovery of target and non-target muscles following BTX-A treatment remains unknown. Currently, BTX-A treatment protocols and the timing between repeat injections, are typically based on clinical examination and functional assessment, but not on systematic evaluations of recovery of the structural integrity of the injected muscles.

Therefore, the main purpose of this study was to investigate deterioration and recovery of quadriceps muscles on New Zealand white (NZW) rabbits following a six months, repeat BTX-A injection protocol.

2. Methods

2.1. Experimental design

Twenty seven skeletally mature, one year old, female NZW rabbits were used for this study with approval from the Animal Care Committee of the University of Calgary. Rabbits were allowed normal cage ($65 \times 45 \times 30$ cm^3) activity and received a standard diet.

Rabbits were divided into five groups as follows:

(1) Control group—saline injection unilaterally ($n=5$; Control).
(2) Six-months of repeated monthly BTX-A injections unilaterally+0 months recovery ($n=5$; BTX-A+0 M).
(3) Six-months of repeated monthly BTX-A injections unilaterally+1 month recovery ($n=5$; BTX-A+1 M).
(4) Six-months of repeated monthly BTX-A injections unilaterally+3 months recovery ($n=5$; BTX-A+3 M).
(5) Six-months of repeated monthly BTX-A injections unilaterally+6 months recovery ($n=7$; BTX-A+6 M).

2.2. BTX-A injection protocol

Rabbits were injected with *Clostridium botulinum* type-A neurotoxin complex (BOTOX, Allergan, Inc., Toronto, Ontario, Canada), which was reconstituted with 0.9% sodium chloride to a concentration of 20 U/ml. Rabbits received intramuscular BTX-A injections (3.5 U/kg) randomized to the right or left quadriceps, which were identified by manual palpation and divided into superior and inferior halves, and into a medial, central and lateral section. One sixth of the total BTX-A dose was equally divided and injected into each section (Longino et al., 2005a, 2005b).

Control group rabbits received intramuscular saline injections of equal volume as that of BTX-A injected experimental group rabbits. BTX-A experimental groups 2 to 5 (BTX-A+0 M/1 M/3 M/6 M) received intramuscular monthly BTX-A injections into the same hindlimb (randomized for the first injection) for a six months period. Additionally, BTX-A experimental groups 3 to 5 (BTX-A+1 M/3 M/6 M) were allowed to recover for periods of 1, 3, and 6 months, respectively before sacrifice.

The primary outcome measurements were knee extensor strength, quadriceps muscle mass, and percentage of contractile material in the injected and contralateral non-injected quadriceps. Additionally, the long-term effects of BTX-A injections were assessed using a glycogen depletion protocol and knee extensor strength measurements with direct muscle stimulation in BTX-A+6 M group rabbits.

2.3. Knee extensor strength, glycogen depletion protocol, and muscle mass

Knee extensor strength was measured at 0, 1, 3, and 6 months following the BTX-A treatment period (BTX-A+0 M/1 M/3 M/6 M) in injected and contralateral non-injected quadriceps using femoral nerve stimulation. Rabbits were secured in a stereotactic frame using metal pins at the pelvis and femoral condyles. Isometric knee extensor forces at 100° of knee flexion (full knee extension was defined as 180°) were measured using a strain-gauged bar placed over the anterior and distal portion of the rabbit's tibia (Longino et al., 2005a, 2005b).

Stimulation of the knee extensor musculature (Grass S8800 stimulator; Astro-Med Inc., Longueil, Quebec, Canada) was given 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, pulse duration 0.1 ms, and the frequency of stimulation was 100 Hz.

Additionally, knee extensor strength was also measured by means of direct muscle stimulation of the injected and contralateral non-injected quadriceps muscles in BTX-A+6 M group rabbits. Rabbits were secured as described above with self-adhesive electrodes placed in the mid-belly of the quadriceps group, and maximum force was obtained using a supra-maximal stimulation at 100 Hz.

Following strength assessment, BTX-A+6 M group rabbits were subjected to a bilateral quadriceps glycogen depletion protocol using fifteen minutes of continuous femoral nerve stimulation at 20 Hz (Shaari et al., 1991). Following glycogen depletion, rabbits were sacrificed and the quadriceps musculature was excised bilaterally. Wet quadriceps muscle mass was determined using a commercial scale (0.001 g). Subsequently, the central third portion of the quadriceps musculature was embedded in paraffin and cut cross-sectionally with a microtome (Leica RM 2165). For every 100 μm, an 8 μm section was collected for glycogen staining using a periodic acid-Schiff (PAS) assay (Sigma-Aldrich, USA). Photographs were taken using an Axionstar *plus* microscope (Carl Zeiss) at 5× magnification. Positive and negative controls for PAS staining were established using quadriceps muscles from Control group rabbits (Fig. 1).

2.4. Percentage of contractile material

The contractile material was defined as the percentage of contractile material tissue quantified in histological samples of the quadriceps musculature. In preparation for histology, the quadriceps musculature was harvested, fixed, cut, and photographed as described above for the glycogen depletion protocol, except that sections were stained with haematoxylin–eosin (H&E) (Leica ST5010). H&E stains the contractile material red and the non-contractile material (primarily connective tissue and fat) white. Five histological sections representing at least 50% of the total muscle cross-sectional area were used to calculate the percentage of contractile material relative to the total cross-sectional area of the muscles.

2.5. Data analysis

Knee extensor strength and muscle mass of the injected and contralateral non-injected muscles of experimental group rabbits were expressed as a percentage of the values obtained in Control group rabbits. Reduction in strength will hereafter