A Clinically Relevant BTX-A Injection Protocol Leads to Persistent Weakness, Contractile Material Loss, and an Altered mRNA Expression Phenotype in Rabbit Quadriceps Muscles

Original Article, Submitted to Journal of Biomechanics

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Keywords: Botulinum toxin, muscle weakness, muscle atrophy, fibrosis, spasticity, cerebral palsy.

Abstract word count: 219

Abstract

Botulinum toxin type-A (BTX-A) injections have become a common treatment modality for patients suffering from muscle spasticity. Despite its benefits, BTX-A treatments have been associated with adverse effects on target muscles. Currently, application of BTX-A is largely based on clinical experience, and research quantifying muscle structure following BTX-A treatment has not been performed systematically. The purpose of this study was to evaluate strength, muscle mass, and contractile material six months following a single or repeated (2 and 3) BTX-A injections into the quadriceps femoris of New Zealand white rabbits. Twenty three skeletally mature rabbits were divided into four groups: experimental group rabbits received 1, 2, or 3 injections at intervals of 3 months (1-BTX-A, 2-BTX-A, 3-BTX-A, respectively) while control group rabbits received volume-matched saline injections. Knee extensor strength, quadriceps muscle mass, and quadriceps contractile material of the experimental group rabbits were expressed as a percentage change relative to the control group rabbits. One-way ANOVA was used to determine group differences in outcome measures (α=0.05). Muscle strength and contractile material were significantly reduced in experimental compared to control group rabbits but did not differ between Experimental groups. Muscle mass was the same in experimental BTX-A and control group rabbits. We concluded from these results that muscle strength and contractile material does not fully recover within six months of BTX-A treatment.

1 Introduction

2 Botulinum toxin type-A (BTX-A) injections have become a common treatment modality for an

increasing number of neuromuscular disorders with the primary aim to relax spastic muscles, for example, in patients with cerebral palsy or following a stroke(Bakheit et al., 2001; Koman et al., 1993; Koman et al., 2001; Molenaers et al., 2006). Once injected into the target muscles, BTX-A prevents acetylcholine release at the motor nerve endings, thereby producing a dose-dependent muscle paralysis (Simpson, 2004).

The positive effects of BTX-A injection in reducing muscle spasticity are well described in the literature: BTX-A injections have been shown to reduce muscle tone, thereby improving the range of motion and functionality of joints (Cardoso et al., 2006; Koman et al., 2000; Koman et al., 2001). As a result, aggressive surgical intervention can often be delayed (Molenaers et al., 2006). Typically, BTX-A injections are repeated at 3-6 month intervals due to the reversible and time limited action of BTX-A, thereby prolonging the period of reduced spasticity(Graham et al., 2000; Heinen et al., 2010).

Despite its beneficial effects, BTX-A treatment has not been approved by the US Food and Drug Administration (FDA) for cerebral palsy children due to the unknown risks associated with the use of BTX-A treatments for already weakened patients (Frasson et al., 2012; Albavera-Hernandez et al., 2009; Ansved et al., 1997; Eleopra et al., 1996; Garner et al., 1993; Naidu et al., 2010). Specifically, Schroeder et al reported significant muscle atrophy of healthy human gastrocnemius lateralis one year following a single, clinically relevant BTX-A injection, suggesting that BTX-A induced atrophy and muscle weakness may last well beyond the expected

treatment period of BTX-A (Schroeder et al., 2009). Additionally, we demonstrated that an aggressive BTX-A injection protocol for six months led to substantial muscle weakness, muscle atrophy, and loss of contractile material in rabbit quadriceps muscles, and that muscles did not fully recover within a six month period following the last BTX-A injection (Fortuna et al., 2011; Fortuna et al., 2013a). However, the first study was a pilot experiment involving only two subjects, while the latter used an aggressive BTX-A treatment protocol not reflecting clinical practice. Currently, clinical application of BTX-A, and the timing between repeat injections, are based on clinical experience and anecdotal evidence, and research quantifying muscle strength and structural damage following a recovery period after BTX-A treatment has not been performed systematically.

Therefore, the purpose of the present study was to evaluate strength, muscle mass, contractile material, and selected mRNA expression profiles six months following a single or repeated (2 and 3) BTX-A injections. The rabbit quadriceps femoris musculature was chosen as an appropriate pre-clinical model and intervals of 3 months were chosen between repeat injections to approximate clinically accepted practice**.**

Methods

Experimental design

Twenty three skeletally mature, one year old, female NZW rabbits were used for this study. All procedures were approved by the Animal Care Committee of the University of Calgary. Rabbits were allowed normal activity in a 65 x 45 x 30cm^3 cage, and received a standard diet.

Rabbits were divided into four groups:

(1) Control group – saline injection unilaterally (n=5; Control)

(2) Single Botox injection unilaterally (n=6; 1-BTX-A)

(3) Two Botox injections with a 3 month interval between injections (n=6; 2-BTX-A)

(4) Three Botox injections with a 3 month interval between injections (n=6; 3-BTX-A).

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 at a total dose of 3.5 U/kg. Injections were randomized to either the right or left quadriceps. The anterior compartment of the thigh was isolated by manual palpation and the quadriceps was visually divided into superior and inferior halves. Each half was subdivided into a medial, central and lateral section. One sixth of the total BTX-A dose was injected into each section to increase its diffusion and to equally distribute the toxin throughout the different portions of the quadriceps musculature (Longino et al., 2005a; Longino et al., 2005b).

Control group 1 received randomized intramuscular saline injections. The total volume of injected saline was the same as the total volume of BTX-A injected into the experimental group animals. Group 2 received a single intramuscular BTX-A injection and was evaluated six months post-injection. Groups 3 and 4 received two and three BTX-A injections, respectively, with a three

month interval between injections, and rabbits were evaluated six months following the last injection.

The primary outcome measures were the isometric knee extensor torque (measured via femoral nerve stimulation and via direct muscle stimulation), the mass of the individual quadriceps muscles, the percentage of contractile material, and select mRNA expression profiles in the target muscles.

Determination of knee extensor strength and muscle mass:

Isometric knee extensor strength was measured in the BTX-A injected musculature six months following the last BTX-A injection. Knee extensor strength was assessed by stimulating the quadriceps via a femoral nerve cuff electrode implanted prior to testing. Following nerve cuff implantation, rabbits were secured in a stereotactic frame using bone pins at the pelvis and femoral condyles. Isometric knee extensor forces at 100˚ of knee flexion were measured using a strain-gauged, calibrated bar placed over the distal portion of the rabbit's tibia (Longino et al., 2005b).

Stimulation of the knee extensor musculature (Grass S8800 stimulator; Astro-Med Inc., Longueil, Quebec, Canada) was performed at a voltage three times higher than the alpha motoneuron threshold, to ensure activation of all motor units (Herzog and Leonard, 1997). Stimulation duration was 500ms, pulse duration 0.1ms, and the frequency of stimulation was 100Hz.

Knee extensor strength was also measured by means of direct muscle stimulation of the injected musculature in the BTX-A experimental group rabbits. Rabbits were positioned as described above. The quadriceps musculature was identified and self-adhesive superficial electrodes were placed over the mid-belly region. Maximum forces were obtained by increasing the voltage (frequency at 100Hz) of stimulation until no further increases in force were detected (Fortuna et al., 2013a; Fortuna et al., 2013b).

Following knee extensor strength assessment, animals were sacrificed by an overdose of Euthanyl (MTC Pharmaceutical; Cambridge, Ontario) into the lateral ear vein. Wet mass for the individual quadriceps femoris muscles was determined using a commercial scale with a resolution of 0.001g.

Determination of the percentage of contractile material:

The percentage of contractile material was determined histologically. The central third of the quadriceps muscles was embedded in paraffin (automatic paraffin processor, Leica TP 1020) and cut cross-sectionally with a microtome (Leica RM 2165). For every 100µm, an 8µm section was collected for staining with haematoxylin-eosin (H&E) (Leica ST5010). Five slides were analyzed and averaged for each muscle (Fortuna et al., 2011).

Photographs were taken from each section using an Axionstar plus microscope (CarlZeiss) with a 5X magnification objective. A customized MatLab program (MatLab 7.8, R2010b) was used to calculate the percentage of contractile material for at least 50% of the total cross-sectional area of each muscle.

RNA isolation and RT-qPCR analysis of muscle tissue:

Samples of frozen quadriceps femoris tissue were powdered at liquid nitrogen temperatures with a Braum Mikro-dismembrator (B. Braum Biotech International, Allentown, PA, USA) and total RNA isolated using the TriSpin method (Reno et al., 1997). Briefly, following powdering, the samples were treated with Trizol Reagent (Life Technologies, Gaithersburg, MD, USA) and the RNA-rich layer was further fractionated and then treated with DNAase, as described previously (Hart and Achari, 2010; Leumamn et al., 2012). Total RNA was quantified using a NanoVue Spectrophotometer (GE Healthcare, Baie d'Urfe, Quebec).

Aliquots (1µg) of each sample were then reverse transcribed using an Omniscript RT Kit (Qiagen Inc., Chatsworth, Calif., USA). All samples in a given set were reverse transcribed at the same time to avoid potential variation. qPCR was performed as described previously (Hart and Achari, 2010) with an iCycler (BioRad Laboratories Inc, Mississauga, ON) and validated using rabbit-specific primer sets for the molecules listed in Table 1. All assessments were performed in duplicate or triplicate under optimal conditions that conformed to qPCR criteria.

Data analysis:

Knee extensor strength (femoral nerve stimulation) and muscle mass of BTX-A injected groups were expressed as a percentage of the values obtained from control group rabbits. Knee extensor strength for direct muscle stimulation of BTX-A injected animals was expressed as the change in strength relative to the corresponding values obtained from femoral nerve stimulation. The amount

of contractile material for each muscle is given as the percentage area of the contractile material relative to the total cross-sectional area of the analyzed sections.

A one-way ANOVA was used to asses muscle weakness, atrophy, contractile material, and mRNA profiles with the main factor groups (Control, 1-BTX-A, 2-BTX-A, and 3-BTX-A). A post-hoc Tukey test was performed if indicated. A paired t-test was used for comparisons between femoral nerve vs. direct muscle stimulation on BTX-A experimental group rabbits. The level of significance was chosen as $\alpha=0.05$ a priori.

Results

Muscle strength:

Muscle strength was significantly ($p<0.05$) reduced to 45% at six months following a single BTX-A injection compared to control (100% - white bar; Fig. 1). There was no additional loss in muscle strength for repeat BTX-A injections (2/3-BTX-A group rabbits; $p>0.05$).

Muscle mass:

Muscle mass in all experimental BTX-A group animals was unaltered compared to Control group rabbits at six months following the last injection ($p>0.05$; Fig. 2).

Percentage of contractile material:

The percentage of contractile material for Control rabbits was 96.9±2.0% (Fig. 3). Following a single BTX-A injection, the contractile material was reduced to 59.2±6.0% at six months following

the injection. There was no additional loss in contractile material at six months following the last injection for rabbits receiving 2 injections (62.5±6.1%) or 3 injections (59.9±11.8%).

Femoral nerve vs. direct muscle stimulation in the 3-BTX-A group rabbits:

Direct muscle stimulation of the injected quadriceps of 3-BTX-A group rabbits produced 15% greater quadriceps force than femoral nerve stimulation ($p<0.05$; Fig. 4).

mRNA expression profiles:

Experimental BTX-A group rabbits (1-BTX-A, 2-BTX-A, and 3-BTX-A) showed significantly elevated mRNA levels for fibrotic scar tissue and muscle inflammatory markers (Fig. 5). Specifically, mRNA expression levels for the matrix molecules collagen I and III, the anabolic growth factors IGF-1 and TGFß, and muscle-specific atrophy marker MuRF1 were significantly elevated in the BTX-A injected animals compared to values in control group rabbits. There were no statistically significant differences in mRNA profiles between the three experimental group rabbits.

Discussion

The primary results of this study were that BTX-A injections resulted in strength loss, structural damage and altered mRNA profiles for a select subset of relevant molecules in the rabbit quadriceps muscles six months following a clinically relevant treatment protocol. Furthermore, and somewhat surprisingly, these adverse effects were not exacerbated by multiple injections of BTX-A.

Muscle strength was significantly decreased despite the six months recovery period following the last BTX-A injection (Fig. 1). The persistence of muscle weakness and contractile material loss suggests that BTX-A injections produce effects that last much longer than previously thought. Currently, a 3-6 months interval between BTX-A injections is recommended (Graham et al., 2000) to prevent antibody responses against the toxin and to avoid cumulative damage of the target muscles. In a recent paper, it had been shown that a single BTX-A injection into a healthy human muscle caused a loss in muscle volume one year following the injection. Furthermore, patients suffering from anterior knee pain who received BTX-A injections into the vastus lateralis musculature reported improved function and reduced knee pain up to 2 years following treatment. Combined with our results, these findings suggest that a clinically relevant BTX-A treatment protocol may produce effects that are much longer lasting than previously thought (Kanovsky et al., 2009; Hasting-Ison et al., 2013). Therefore, it might be prudent to carefully evaluate the frequency of BTX-A injections, especially in patients who already have a weakened musculature, such as children with cerebral palsy (Damiano et al., 2001; Damiano and Moreau, 2008; Stackhouse et al., 2007; Wiley and Damiano, 1998).

Muscle strength did not recover to Control values following a six months recovery period from the last BTX-A injection. This result is likely caused by the reduced amount of contractile material following the recovery period (Fig. 3), and the remnant blockage of acetylcholine at the neuromuscular junction, as evidenced by the greater quadriceps femoris forces obtained with direct muscle stimulation compared to femoral nerve stimulation (Fig. 4). Direct muscle stimulation increased peak quadriceps forces across all experimental groups by 15%, on average. Combined with the 34% average loss of contractile material, which would cause at least a 34% in force loss,

the average loss of muscle strength of 45% across all experimental group rabbits is well explained by the remnant BTX-A effect and the loss of contractile material (Fig. 6). From the results of this study, it is not clear how long it might take for the contractile material, and thereby muscle strength, to return to Control values. It is perceivable that BTX-A alters muscle repair mechanisms permanently, thereby reaching a new homeostatic state, as evidenced by the altered mRNA expression six months following the last injection. Studies using a one or two year recovery period should be performed to evaluate if muscles ever return to normal control values following BTX-A treatment.

Muscle mass was the same across all experimental and control group rabbits. At first glance, this result may be surprising. However, careful analysis revealed that the loss of contractile material was offset by a gain in non-contractile material, primarily fat, thereby leaving the overall muscle mass, but not the muscle composition, approximately constant. This finding might be of clinical relevance, as muscle volume measured using non-invasive imaging techniques (MRI, ultrasound) are sometimes used to approximate muscle mass in patient populations to determine progression of a disease or success of a treatment intervention (Damiano and Moreau, 2008). Structural integrity and functional properties of muscles, rather than muscle mass or volume, might be more appropriate outcome measures to determine disease progression or intervention effects.

Increasing the number of injections did not produce additional loss in muscle strength and contractile material, as one might have suspected, suggesting that most of the adverse effects of BTX-A injection into muscles are caused by the first injection, or that the recovery period between injections, was sufficient for partial recovery, thereby offsetting the potential damage induced by

each injection. Antibody responses following first BTX-A exposure may prevent muscles from additional damage to subsequent exposures to the toxin, and such responses should be measured in future experiments.

Our findings agree with those of Barber et al. who found no differences in muscle volume, fascicle length, and physiological cross-sectional area in patients with spastic cerebral palsy exposed to single and repeat BTX-A injections (Barber et al., 2013). Children with spastic cerebral palsy already have a weakened musculature and a loss of muscle mass (Damiano and Moreau, 2008; Damiano and Moreau, 2008; Moreau et al., 2010; Moreau et al., 2012), therefore submitting them to BTX-A treatments has to be evaluated carefully using a risk and benefit assessment, and although relieve from spasticity and increased joint range of motion in the short term, might be offset by a loss in muscle mass and strength in the long term.

The temporary blockage effects of BTX-A treatments are intended to reduce muscle spasticity, thereby offering a “window of opportunity” to re-train target muscles and induce improved motor control and muscle coordination with minimal adverse effects. Therefore, BTX-A treatments should be combined with an exercise protocol aimed at taking full advantage of the temporary relaxation of the spastic musculature. An exercise protocol during BTX-A treatment can help alleviate muscle atrophy and weakness, which are adverse effects of BTX-A treatments. It has been shown that direct muscle stimulation in conjunction with BTX-A treatments limits the loss of contractile material and retains strength and function of muscles by circumventing the loss of voluntary muscle activation caused by BTX-A induced nerve ablation (Fortuna et al., 2013b; Williams et al., 2013). Our findings suggest that multiple injections do not produce cumulative

adverse effects in muscle structure, composition and function, suggesting that patients can benefit from multiple injections, thereby prolonging the period of improved joint function and reduced spasticity which might help delay invasive surgical interventions and might provide increased independence of patients with cerebral palsy. The fact that a single BTX-A injection can induce significant muscle atrophy and weakness should not be seen as a positive outcome. Long term muscle weakness in already weakened cerebral palsy children can further compromise joint function and quality of life. Hence, long-term follow-up studies should be encouraged to assess muscle structure and joint function during and after BTX-A treatments in children with cerebral palsy.

Following BTX-A injections, there is a persistent mRNA elevation for fibrotic response molecules (Hart, 2013). While we did not detect overt fibrosis in our study, a persistent low grade fibrotic response may interfere with normal muscle repair, consistent with the idea of compromised muscle function. Future investigations should focus on elucidating the mechanisms of repair in muscles exposed to BTX-A.

Conclusions

We conclude from the results of this study that muscle strength and contractile material do not fully recover from BTX-A exposure within a six months recovery period. Furthermore, increased frequency of BTX-A exposure was not associated with increased adverse effects in the target muscles.

Acknowledgments:

The authors thank CAPES Brazil, The NSERC-CIHR Collaborative Health Research Program, the Cerebral Palsy International Research Foundation, the Canada Research Chair Programme, the International Society of Biomechanics, the McCaig Professorship, the AIHS OA Team Grant, and the Killam Foundation for financial assistance. We would like to thank Dr. Tak Fung for assistance with the statistical analysis.

REFERENCES

Albavera-Hernandez, C., Rodriguez, J. M., Idrovo, A. J., 2009. Safety of botulinum toxin type A among children with spasticity secondary to cerebral palsy: a systematic review of randomized clinical trials. Clinical Rehabilitation 23, 394-407.

Ansved, T., Odergren, T., Borg, K., 1997. Muscle fiber atrophy in leg muscles after botulinum toxin type A treatment of cervical dystonia. Neurology 48, 1440-1442.

Bakheit, A. M., Pittock, S., Moore, A. P., Wurker, M., Otto, S., Erbguth, F., Coxon, L., 2001. A randomized, double-blind, placebo-controlled study of the efficacy and safety of botulinum toxin type A in upper limb spasticity in patients with stroke. European Journal of Neurology : The Official Journal of the European Federation of Neurological Societies 8, 559-565.

Barber, L., Hastings-Ison, T., Baker, R., Kerr Graham, H., Barrett, R., Lichtwark, G., 2013. The effects of botulinum toxin injection frequency on calf muscle growth in young children with spastic cerebral palsy: a 12-month prospective study. Journal of Children's Orthopaedics 7, 425433.

Cardoso, E. S., Rodrigues, B. M., Barroso, M., Menezes, C. J., Lucena, R. S., Nora, D. B., Melo, A., 2006. Botulinum toxin type A for the treatment of the spastic equinus foot in cerebral palsy. Pediatric Neurology 34, 106-109.

Damiano, D. L., Moreau, N., 2008. Muscle thickness reflects activity in CP but how well does it represent strength? Developmental Medicine and Child Neurology 50, 88

Damiano, D. L., Quinlivan, J., Owen, B. F., Shaffrey, M., Abel, M. F., 2001. Spasticity versus strength in cerebral palsy: relationships among involuntary resistance, voluntary torque, and

motor function. European Journal of Neurology : The Official Journal of the European Federation of Neurological Societies 8 Suppl 5, 40-49.

Dunne, J. W., Singer, B. J., Silbert, P. L., Singer, K. P., 2010. Prolonged vastus lateralis denervation after botulinum toxin type A injection. Movement Disorders : Official Journal of the Movement Disorder Society 25, 397-401.

Eleopra, R., Tugnoli, V., Caniatti, L., De Grandis, D., 1996. Botulinum toxin treatment in the facial muscles of humans: evidence of an action in untreated near muscles by peripheral local diffusion. Neurology 46, 1158-1160.

Fortuna, R., Horisberger, M., Vaz, M. A., Herzog, W., 2013a. Do skeletal muscle properties recover following repeat onabotulinum toxin A injections? Journal of Biomechanics 46, 24262433.

Fortuna, R., Horisberger, M., Vaz, M. A., Van der Marel, R., Herzog, W., 2013b. The effects of electrical stimulation exercise on muscles injected with botulinum toxin type-A (botox). Journal of Biomechanics 46, 36-42.

Fortuna, R., Vaz, M. A., Youssef, A. R., Longino, D., Herzog, W., 2011. Changes in contractile properties of muscles receiving repeat injections of botulinum toxin (Botox). Journal of Biomechanics 44, 39-44.

Frasson, E., Dall'ora, E., Bordignon, M., Brigo, F., Tocco, P., Primon, D., Didone, G., Vicentini, S., Fiaschi, A., Bertolasi, L., 2012. Spread of botulinum neurotoxin type a at standard doses is inherent to the successful treatment of spastic equinus foot in cerebral palsy: short-term neurophysiological and clinical study. Journal of Child Neurology 27, 587-593.

Garner, C. G., Straube, A., Witt, T. N., Gasser, T., Oertel, W. H., 1993. Time course of distant effects of local injections of botulinum toxin. Movement Disorders : Official Journal of the Movement Disorder Society 8, 33-37.

Graham, H. K., Aoki, K. R., Autti-Rämö, I., Boyd, R. N., Delgado, M. R., Gaebler-Spira, D. J., Gormley, M. E., Guyer, B. M., Heinen, F., Holton, A. F., Matthews, D., Molenaers, G., Motta, F., García Ruiz, P. J., Wissel, J., 2000. Recommendations for the use of botulinum toxin type A in the management of cerebral palsy. Gait & Posture 11, 67-79.

Hart, D. A., Achari, Y., 2010. Alterations to cell metabolism in connective tissues of the knee after ovariohysterectomy in a rabbit model: are there implications for the postmenopausal athlete? British Journal of Sports Medicine 44, 867-871.

Hastings-Ison T, Rawicki B, Baker R, Blackburn C, Fahey M, Simpson P, Graham H. Determining the optimum frequency of Botulinum toxin injections to the gastrocsoleus in children with cerebral palsy - an RCT. Dev Med Child Neurol 2013;55(s3):38.

342 Heinen, F., Desloovere, K., Schroeder, A. S., Berweck, S., Borggraefe, I., van Campenhout, A.,
343 Andersen, G. L., Aydin, R., Becher, J. G., Bernert, G., Caballero, I. M., Carr, L., Valayer, E. C.,
344 Desiato, M. T., Fairhurst, C., Filipetti, P., Hassink, R., Hustedt, U., Jozwiak, M., Kocer, S. I.,
345 Kolanowski, E., Krägeloh-Mann, I., Şehim Kutlay, Mäenpää, H., Mall, V., McArthur, P., Morel,
346 E., Papavassiliou, A., Pascual-Pascual, I., Pedersen, S. A., Plasschaert, F. S., Irene van der Ploeg,
347 Remy-Neris, O., Renders, A., Di Rosa, G., Steinlin, M., Tedroff, K., Valls, J. V., Viehweger, E.,
348 Molenaers, G., 2010. The updated European Consensus 2009 on the use of Botulinum toxin for
349 children with cerebral palsy. European Journal of Paediatric Neurology 14, 45-66.

350 Herzog, W., Leonard, T. R., 1997. Depression of cat soleus-forces following isokinetic shortening.
351 Journal of Biomechanics 30, 865-872.

352 Kanovsky, P., Bares, M., Severa, S., Richardson, A., Dysport Paediatric Limb Spasticity Study
353 Group, 2009. Long-term efficacy and tolerability of 4-monthly versus yearly botulinum toxin
354 type A treatment for lower-limb spasticity in children with cerebral palsy. Developmental
355 Medicine and Child Neurology 51, 436-445.

356

357 Koman, L. A., Brashear, A., Rosenfeld, S., Chambers, H., Russman, B., Rang, M., Root, L.,
358 Ferrari, E., Garcia de Yebenes Prous, J., Smith, B. P., Turkel, C., Walcott, J. M., Molloy, P. T.,
359 2001. Botulinum toxin type a neuromuscular blockade in the treatment of equinus foot deformity
360 in cerebral palsy: a multicenter, open-label clinical trial. Pediatrics 108, 1062-1071.

361 Koman, L. A., Mooney, J. F.,3rd, Smith, B., Goodman, A., Mulvaney, T., 1993. Management of
362 cerebral palsy with botulinum-A toxin: preliminary investigation. Journal of Pediatric
363 Orthopedics 13, 489-495.

364 Koman, L. A., Mooney, J. F.,3rd, Smith, B. P., Walker, F., Leon, J. M., 2000. Botulinum toxin
365 type A neuromuscular blockade in the treatment of lower extremity spasticity in cerebral palsy: a
366 randomized, double-blind, placebo-controlled trial. BOTOX Study Group. Journal of Pediatric
367 Orthopedics 20, 108-115.

368 Leumann, A., Longino, D., Fortuna, R., Leonard, T., Vaz, M., Hart, D.A., Herzog, W., 2012.
369 Altered cell metabolism in tissues of the knee joint in a rabbit model of Botulinum toxin-A
370 induced quadriceps muscle weakness. Scandinavian Journal of Medicine & Science in Sports 22,
371 776-782.

372 Longino, D., Butterfield, T. A., Herzog, W., 2005a. Frequency and length-dependent effects of
373 Botulinum toxin-induced muscle weakness. Journal of Biomechanics 38, 609-613.

374 Longino, D., Frank, C., Leonard, T. R., Vaz, M. A., Herzog, W., 2005b. Proposed model of
375 botulinum toxin-induced muscle weakness in the rabbit. Journal of Orthopaedic Research :
376 Official Publication of the Orthopaedic Research Society 23, 1411-1418.

377 Molenaers, G., Desloovere, K., Fabry, G., De Cock, P., 2006. The effects of quantitative gait
378 assessment and botulinum toxin a on musculoskeletal surgery in children with cerebral palsy.
379 The Journal of Bone and Joint Surgery.American Volume 88, 161-170.

380 Moreau, N. G., Falvo, M. J., Damiano, D. L., 2012. Rapid force generation is impaired in
381 cerebral palsy and is related to decreased muscle size and functional mobility. Gait & Posture 35,
382 154-158.
383
384 Moreau, N. G., Simpson, K. N., Teefey, S. A., Damiano, D. L., 2010. Muscle architecture
385 predicts maximum strength and is related to activity levels in cerebral palsy. Physical Therapy
386 90, 1619-1630.

387 Naidu, K., Smith, K., Sheedy, M., Adair, B., Yu, X., Graham, H. K., 2010. Systemic adverse
388 events following botulinum toxin A therapy in children with cerebral palsy. Developmental
389 Medicine and Child Neurology 52, 139-144.

390 Reno, C., Marchuk, L., Sciore, P., Frank, C. B., Hart, D. A., 1997. Rapid isolation of total RNA
391 from small samples of hypocellular, dense connective tissue. Biotechniques 22, 1082.

392 Schroeder, A. S., Ertl-Wagner, B., Britsch, S., Schroder, J. M., Nikolin, S., Weis, J.,
393 MullerFelber, W., Koerte, I., Stehr, M., Berweck, S., Borggraefe, I., Heinen, F., 2009. Muscle
394 biopsy substantiates long-term MRI alterations one year after a single dose of botulinum toxin
395 injected into the lateral gastrocnemius muscle of healthy volunteers. Movement Disorders :
396 Official Journal of the Movement Disorder Society 24, 1494-1503.

397 Simpson, L. L., 2004. Identification of the major steps in botulinum toxin action. Annual Review
398 of Pharmacology and Toxicology 44, 167-193.

399 Stackhouse, S. K., Binder-Macleod, S. A., Stackhouse, C. A., McCarthy, J. J., Prosser, L. A.,
400 Lee, S. C., 2007. Neuromuscular electrical stimulation versus volitional isometric strength
401 training in children with spastic diplegic cerebral palsy: a preliminary study. Neurorehabilitation
402 and Neural Repair 21, 475-485.

403 Wiley, M. E., Damiano, D. L., 1998. Lower-extremity strength profiles in spastic cerebral palsy.
404 Developmental Medicine and Child Neurology 40, 100-107.

405 Williams, S. A., Elliott, C., Valentine, J., Gubbay, A., Shipman, P., Reid, S., 2013. Combining
406 strength training and botulinum neurotoxin intervention in children with cerebral palsy: the
407 impact on muscle morphology and strength. Disability and Rehabilitation 35, 596-605.

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Figure Legends

Fig 1 Mean muscle strength (±1 SD) normalized to the values of Control group rabbits (100%) six months after the last BTX-A injection in rabbits receiving 0 (Control), 1 (1-BTX-A), 2 (2BTX-A), and 3 (3-BTX-A) botulinum toxin injections with a 3 months interval between injections. Strength in the quadriceps femoris muscles of all experimental group rabbits was reduced compared to control group rabbits, but was the same among the 3 experimental groups. * compared to Control group ($p<0.05$).

Fig 2 Mean muscle mass (±1 SD) normalized to the values of Control group rabbits (100%) six months after the last BTX-A injection in rabbits receiving 0 (Control), 1 (1-BTX-A), 2 (2-BTXA), and 3 (3-BTX-A) botulinum toxin injection with a 3 months interval between injections. Muscle mass in all experimental BTX-A group rabbits was unaltered compared to Control group rabbits ($p>0.05$).

Fig 3 Exemplar histological cross-sectional images showing the percentage of muscle contractile material (H&E – red staining) and non-contractile material (white color – primarily fat and connective tissue). The amount of contractile material for Control group rabbits was 96.9±2.0% (top left). Following a single BTX-A injection, there was a significant reduction of contractile material for 1-BTX-A group rabbits (top right – 59.2±6.0%). There was no additional loss of contractile material for rabbits that received two injections (2-BTX-A; bottom left – 62.5±6.1%) and three injections (3-BTX-A; bottom right – 59.9±11.8%) group rabbits, respectively.

Fig 4 Muscle strength obtained with femoral nerve stimulation (white bar) and by direct muscle stimulation (shaded bar) for the injected quadriceps femoris muscles of 3-BTX-A group rabbits. Strength was normalized relative to Control group values (100%, not shown). Direct muscle

stimulation produced significantly greater muscle forces when compared to femoral nerve stimulation ($p<0.05$). This result suggests that despite a six months recovery period following the last BTX-A injection, there is persistent blockage of acetylcholine release at the neuromuscular junction. * compared to 3 BTX-A femoral nerve stimulation ($p<0.05$).

Fig 5 mRNA expression normalized to the values of Control group rabbits (white bars – 100%). Gene expression of Collagen I, Collagen III, IGF-1, TGFß, and MuRF were significantly increased compared to Control group rabbits. Similar to the mechanical and histological data, there was no difference in mRNA expression between the three experimental groups. * compared to Control group ($p<0.05$).

Fig 6 Average muscle strength in BTX-A group rabbits averaged across all three experimental groups (light gray bar), and estimated loss in strength associated with the reduction in contractile material (dark gray bar), and the remnant effects associated with BTX-A blockage (shaded bar). Six months following the last BTX-A injection, muscle strength was still significantly reduced to 55% of the strength in Control rabbits (white bars). The loss in strength associated with the loss in contractile material was obtained by assuming a linear relationship between the amount of contractile material and strength. This assumption likely underestimates the real loss in strength associated with loss of contractile material.

Figure
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Fig 1

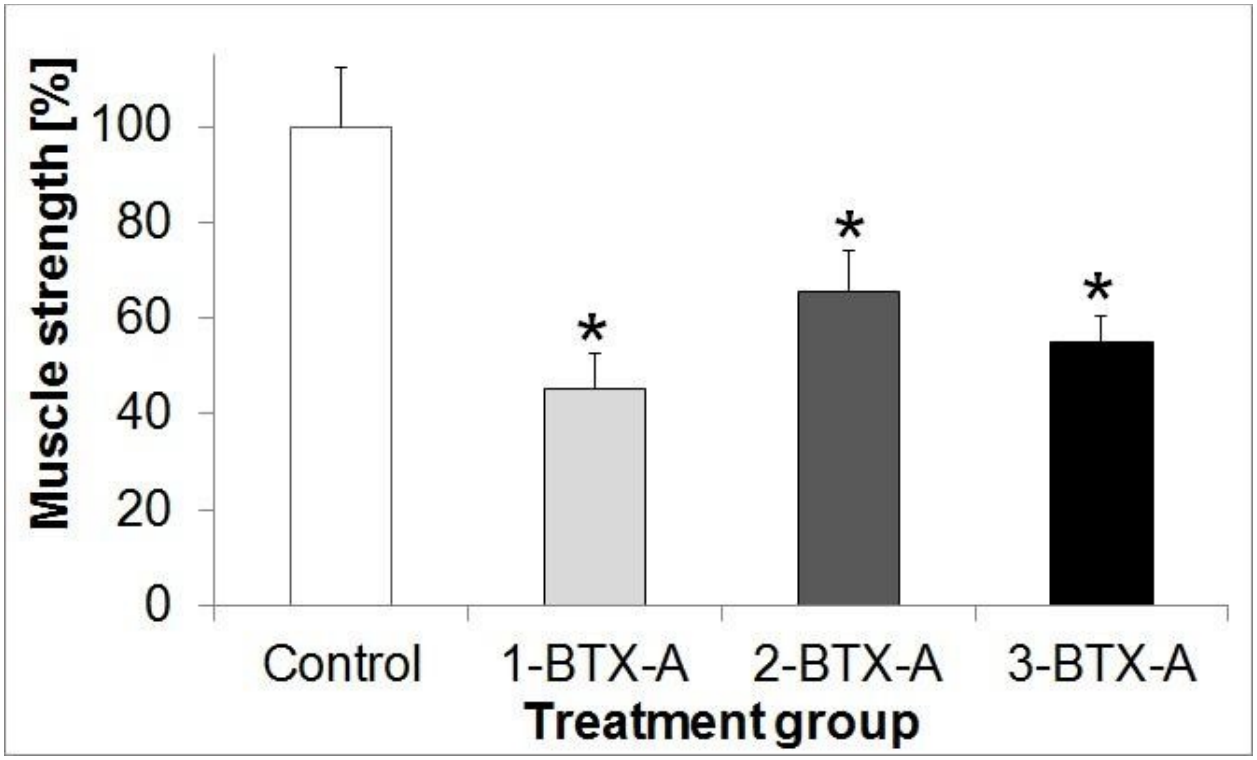

Muscle strength [%]
100
80
60
40
20
0
*
*
*
Control
1-BTX-A
2-BTX-A
3-BTX-A
Treatment group

Fig 2

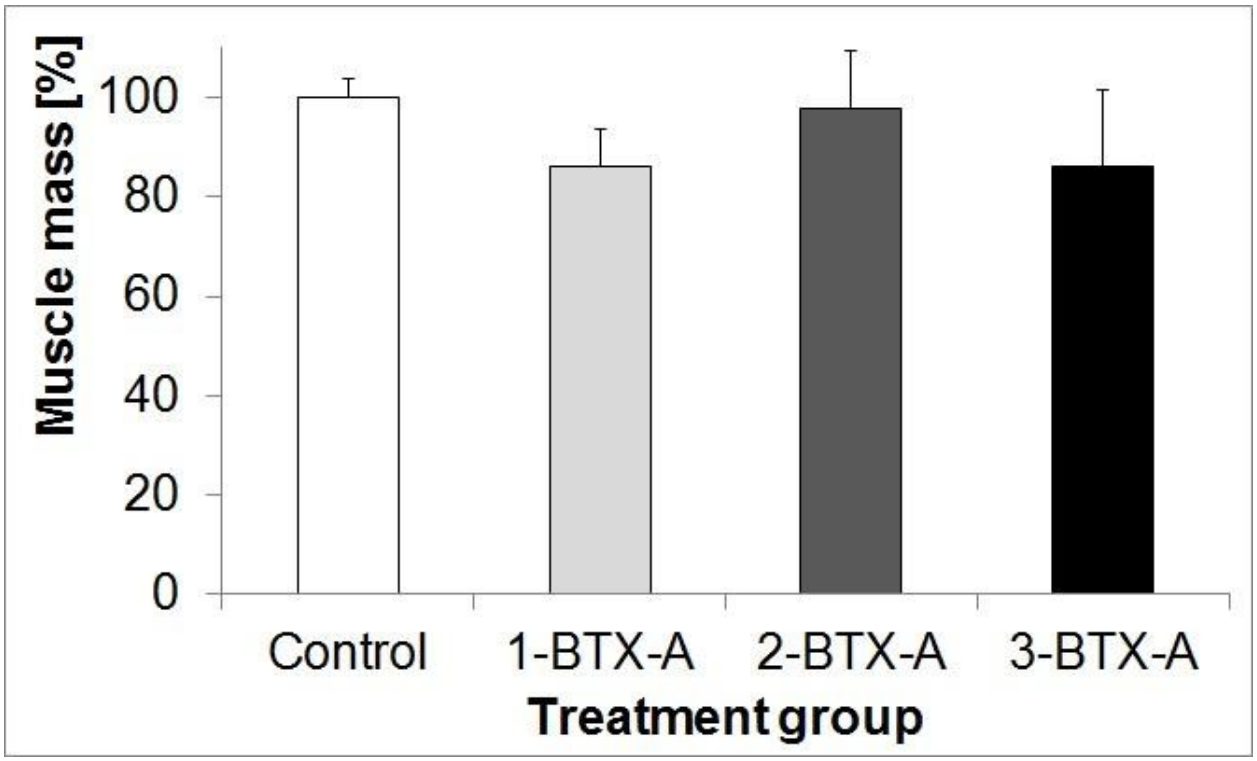

Muscle mass [%]
100
80
60
40
20
0
Control
1-BTX-A
2-BTX-A
3-BTX-A
Treatment group

Fig 3

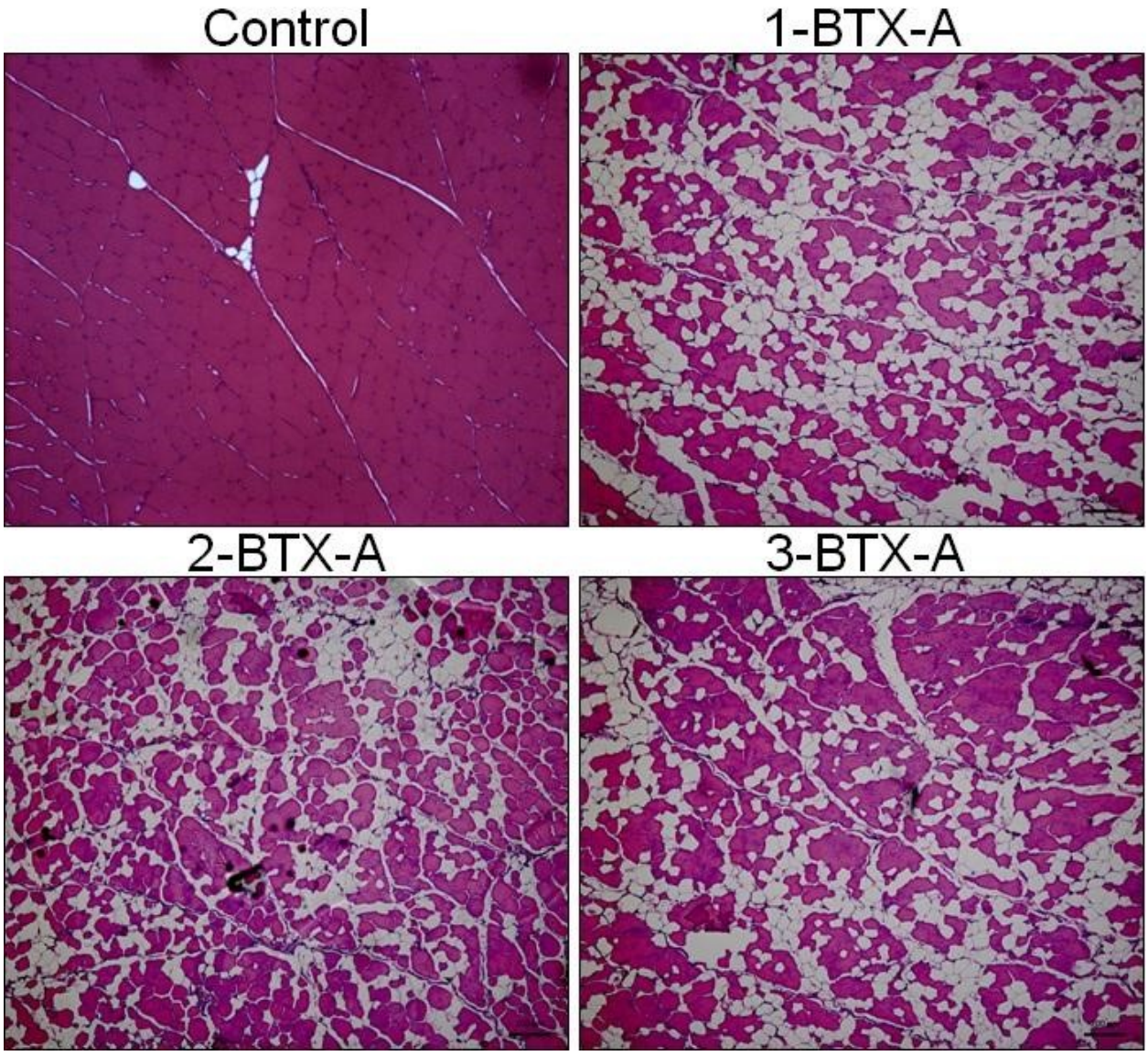


Fig 4

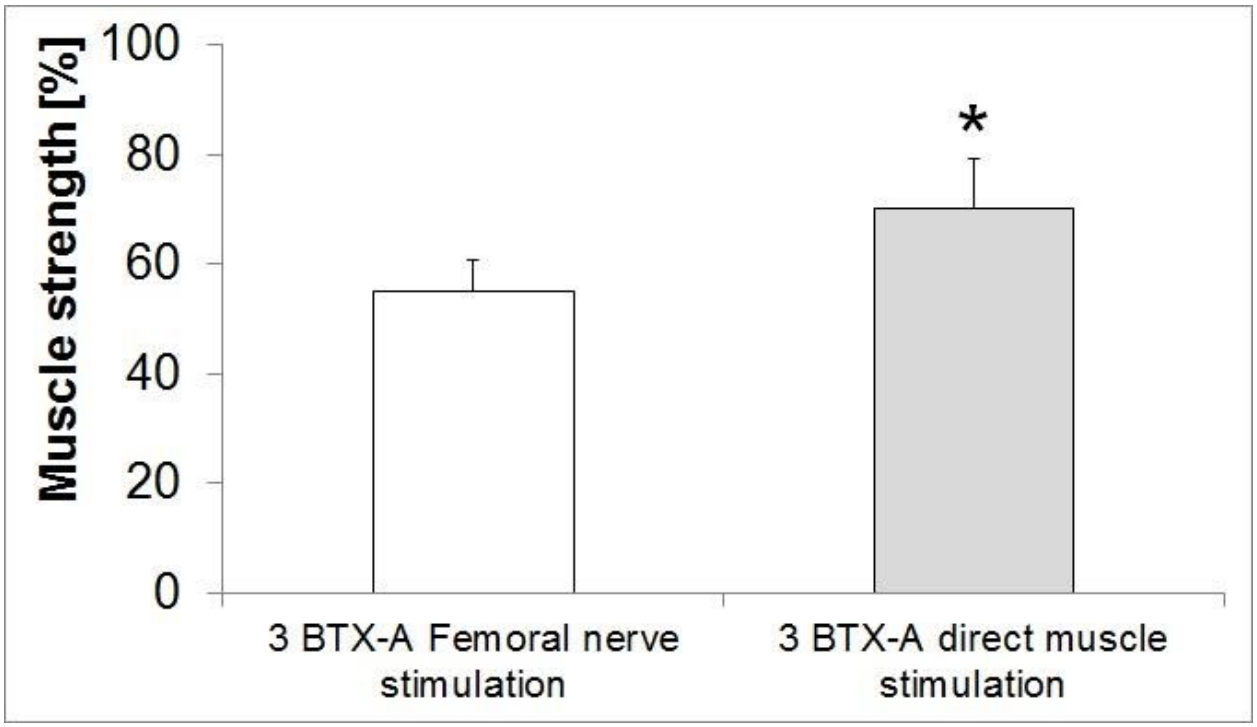

Muscle strength [%]
100
80
60
40
20
0
*
3 BTX-A Femoral nerve stimulation
3 BTX-A direct muscle stimulation

Fig 5

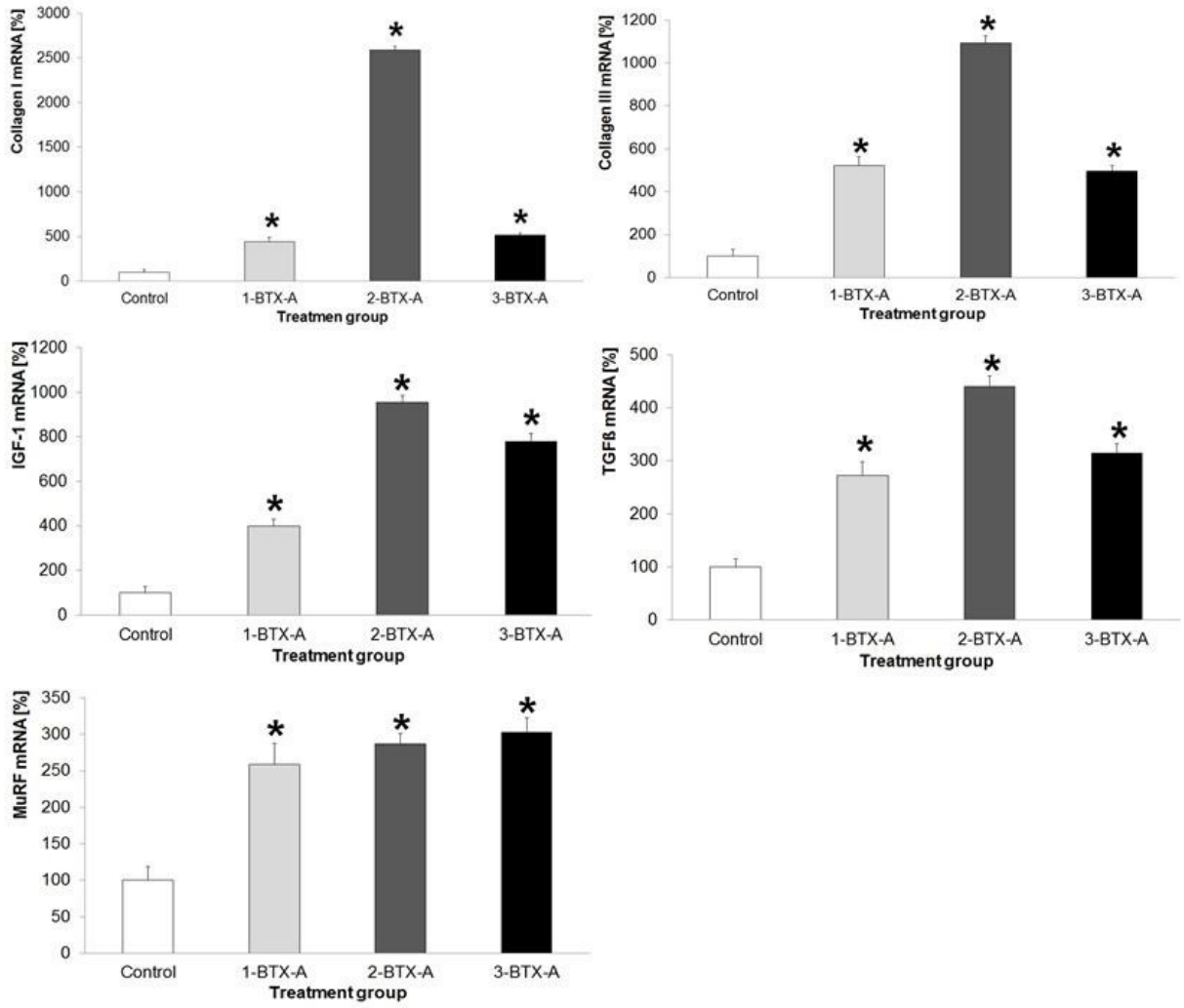
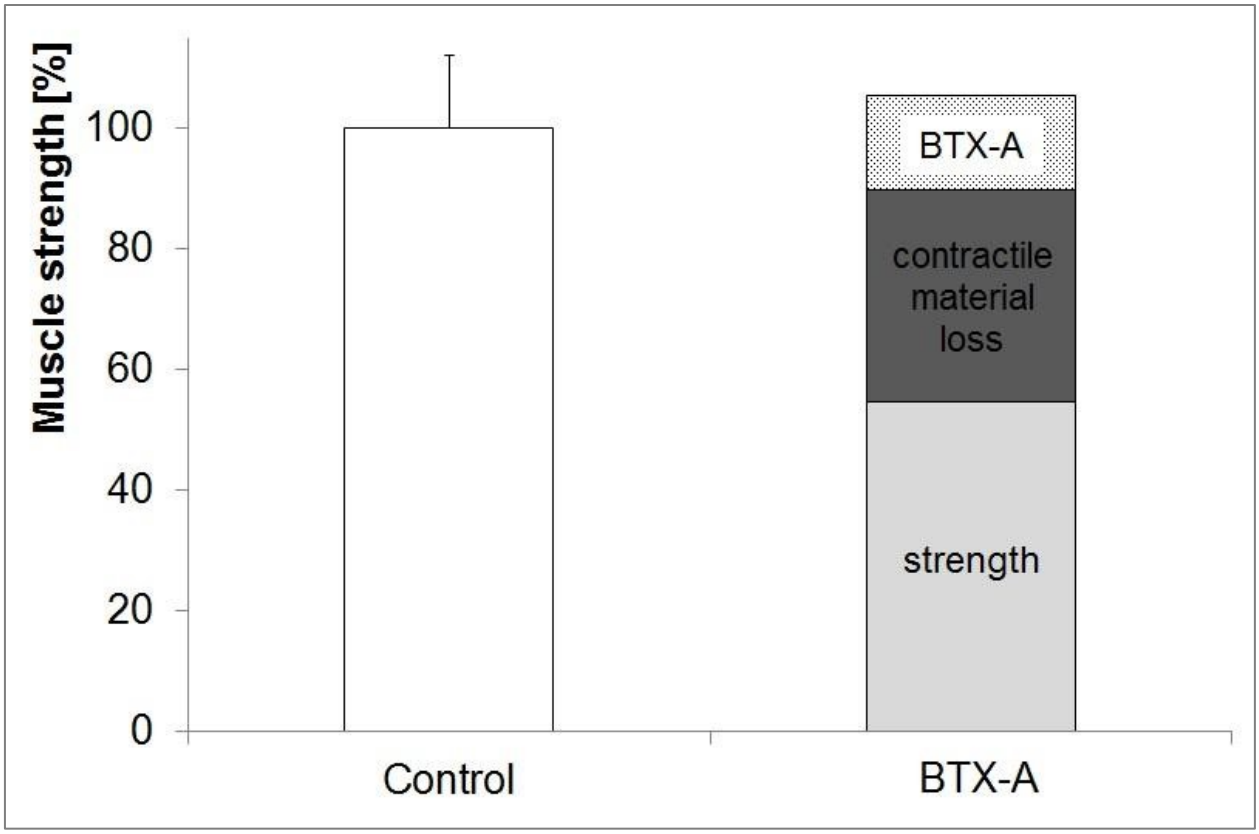

Collagen I mRNA [%]
3000
2500
2000
1500
1000
500
0
Control
1-BTX-A
2-BTX-A
3-BTX-A
Treatmen group
Collagen III mRNA [%]
1200
1000
800
600
400
200
0
Control
1-BTX-A
2-BTX-A
3-BTX-A
Treatment group
IGF-1 mRNA [%]
1200
1000
800
600
400
200
0
Control
1-BTX-A
2-BTX-A
3-BTX-A
Treatment group
TGFß mRNA [%]
500
400
300
200
100
0
Control
1-BTX-A
2-BTX-A
3-BTX-A
Treatment group
MuRF mRNA [%]
350
300
250
200
150
100
50
0
Control
1-BTX-A
2-BTX-A
3-BTX-A
Treatment group

Fig 6


Muscle strength [%]
100
80
60
40
20
0
BTX-A
contractile material loss
strength
Control
BTX-A