Current Topics in Toxicology
Vol. 18, 2022

Original Article

Increased muscle fiber size and pathology with botulinum toxin treatment of upper extremity muscles in cerebral palsy

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ABSTRACT

Intramuscular injections of botulinum toxin A (BTX) are regularly used to treat skeletal muscle spasticity and relieve pain during rehabilitation therapy. However, while numerous preclinical studies have shown dramatic atrophic changes in muscle, little is known about the long-term effect of toxin on human skeletal muscle. In this study, muscle morphology was analyzed in biopsies taken from spastic upper extremity muscles of 8 cerebral palsy patients treated with BTX 5 months to 4 years prior sampling and was compared to muscles from 7 patients who had not ever received BTX treatment (overall 25 muscle biopsies obtained from 6 different muscles.). The most important (and surprising) finding was that BTX-treated muscles contained significantly larger fibers compared to untreated muscles. A strong correlation between fiber size and age was observed but the growth rate in the BTX group was larger. Pathological signs such as central nuclei, neonatal myosin heavy chain expression, angular fibers and hybrid fibers (expressing both slow and fast myosin heavy chain fibers) were significantly greater in BTX-treated muscles compared to untreated muscles. Capillarization was also increased in BTX-treated muscle compared to untreated muscles and was the best predictor of fiber size. We suggest that, in the context of spasticity, BTX may block negative, atrophy-inducing pressure of the central nervous system on skeletal muscle or may allow an altered use pattern that should be considered a positive adjuvant to current rehabilitation therapies.

KEYWORDS: spasticity, neurotoxin, skeletal muscle contracture, myopathy, pathology.

INTRODUCTION

Skeletal muscle spasticity after upper neuron motor lesion can have devastating neural and musculoskeletal consequences. Complications such as joint contracture, pain, skeletal deformity and subluxation often result from traumatic injury such as stroke, head injury or cerebral palsy (CP). Joint contracture treatment may be conservative (e.g., serial casting, splinting, neurotoxin injection or physiotherapy) or surgical. Regardless of the treatment plan, rational approaches to deformity correction must be based, at least in part, on a proper understanding of the secondary changes that occur in spastic skeletal muscle after upper motor neuron lesion.

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There is ample evidence that skeletal muscle subjected to chronic upper motor neuron lesion and concomitant spasticity is abnormal [1]. For example, Sinkjaer *et al*. [2, 3] and Mirbergheri *et al*. [4, 5] used limb perturbation methods to demonstrate changes in the biomechanical properties of plantarflexor muscles in patients with stroke, multiple sclerosis and spinal cord injury. Morphological studies similarly demonstrated abnormalities in muscle fiber type and fiber size distribution in patients with CP [6-8]. Alterations in cellular and extracellular matrix biomechanical properties [9-12] suggest a complex interaction between muscle cells and their extracellular surroundings in response to upper motor neuron lesion. Taken together, spastic skeletal muscle structural and functional properties reveal a complex adaptation that is neither characteristic of the simple increased-use or decreased-use animal models of plasticity [13], but rather represents a unique adaptation to the spastic mechanical and neurological environment.

Neurotoxin treatment alters muscle properties. Neurotoxins exist in various serotypes, where the most commonly used serotype, the type A neurotoxin, is known as Botox® (BTX; Allergan Incorporated, Irvine, California). We use BTX routinely in our clinic to reduce the tone of spastic muscles, to provide patients with the opportunity to 'test' the effects of tendon surgeries (releases, tendon lengthenings, tendon transfers, *etc.*) prior to transfer of spastic muscles, and to permit patient exercise and increased range of motion. Since BTX is said to induce muscle 'reversible chemodenervation' [14-16] it is of interest to determine the response of muscle to BTX injection as well as spasticity. The neurotoxin, that binds to synaptic vesicle protein 2 (SV2) [17], blocks neuromuscular transmission and induces a cycle of sprouting, followed by restoration of the original neuromuscular junction [18]. Thus, if BTX treatment really represents 'reversible chemodenervation,' then the types of changes observed in muscle after BTX injection should be consistent with denervation followed by reinnervation *i.e.,* increased central nuclei, decreased fiber size, and fiber type grouping. While there is a great deal of literature available on the effects of BTX on normal animal muscles such as rat, mouse, dog and rabbit [19-23], there are no studies that have reported morphological changes of spastic human skeletal muscle tissue in response to BTX injection. Since, as described above, human muscle response to upper motor neuron lesion is unique, these pre-clinical models may not fully represent human muscle response to neurotoxin. Thus, the purpose of this study was to quantify the morphological properties of skeletal muscle secondary to spasticity with and without BTX injection. Our goal was to understand the underlying biological processes that occur in BTX-injected spastic skeletal muscles.

MATERIALS AND METHODS

Patient population

Muscle biopsies were obtained from 15 patients (8 males, 7 females) aged between 4 and 20 years. All patients were scheduled for reconstructive surgery for correction of upper extremity contracture. 8 patients had a diagnosis of spastic hemiplegia, 5 spastic diplegia and 2 spastic tetraplegia (Table 1). Of the 15 patients, 7 had never received any BTX-treatment (No BTX, mean age 12.4 ± 5.1 years; mean ± SD, Table 1) and 8 had received BTX-treatment from 5 months to 4 years prior surgery (BTX, mean age 15.1 ± 4.8 years; mean ± SD, Table 1). Thus, the BTX group was about 2.5 years older than the No BTX group which has the potential to confound the results. We tested this possibility explicitly using the statistical analysis described below. The study was approved by the ethical committee of Göteburg University. Patients provided their age-appropriate informed assent and parents provided informed consent for study participation.

Biopsy sampling

During surgery, 25 biopsies were collected from 6 different arm skeletal muscles (Table 1). Samples were carefully taken from the muscle belly, avoiding regions close to the muscle-tendon junction. Samples were immediately transported to the laboratory, oriented under microscope, mounted onto a small piece of cork and snap frozen in isopentane precooled by liquid nitrogen (-196 °C). Samples were stored at -80 ºC until analyzed.

Table 1. Patient characteristics.

Patient number	Gender	Age (year:month)	Diagnosis	Muscle	Treatment
1	M	13:5	Hemiplegia	PT	No BTX
				FCU	BTX
2	M	17:9	Tetraplegia	FCU	BTX
3	F	6:4	Hemiplegia	PT	BTX
4	F	6:5	Diplegia	PT	No BTX
5	M	5:7	Hemiplegia	PT	No BTX
6	F	20:3	Hemiplegia	FCR	BTX
				FCU	BTX
7	F	16:11	Tetraplegia	ADD	No BTX
				FPB	No BTX
				FCU	No BTX
8	M	6:10	Diplegia	PT	BTX
				ADD	BTX
9	M	17:9	Diplegia	FCU	BTX
				PT	BTX
10	F	18:10	Diplegia	Interossei	BTX
11	M	16:11	Hemiplegia	FCU	No BTX
12	M	4:10	Hemiplegia	ADD	No BTX
				PT	No BTX
13	M	13:11	Hemiplegia	ADD	No BTX
				FCU	No BTX
				PT	No BTX
14	F	15:2	Hemiplegia	PT	BTX
				ADD	BTX
15	F	17:3	Tetraplegia	FCU	No BTX

Abbreviations: ADD, adductor pollicis longus; FCR, flexor carpi radialis; FCU, flexor carpi ulnaris; FPB, flexor pollicis brevis; PT, pronator teres.

Histological processing

Transverse serial sections (10 μm) were cut on a cryostat (Microm, Walldorf, Germany), placed onto precoated poly-lysine glass slides (Menzel GmbH & Co, Braunschweig, Germany) and stored at -80 ºC until processing and morphometric analysis. A variety of stains were used to characterize the tissue. Samples for hematoxylin and eosin staining were prefixed for 5 min in Histofix® (Histolab, Gothenburg, Sweden) and subsequently stained. Collagen proportion was evaluated using picrosirius red, a strong anionic dye widely used for staining of collagen in tissue sections [24, 25]. Sections were fixed in Bouin's solution (Histolab, Gothenburg, Sweden) for 30 min, rinsed in distilled H_2O and placed in Sirius Red solution (0.1% in saturated aqueous picric acid, Histolab, Gothenburg, Sweden) for 90 min. A rinse in 0.5 % glacial acid was followed by washing, dehydration and clearing. Sections were then mounted with Pertex® (Histolab, Gothenburg, Sweden).

Immunohistochemical processing

Immunohistochemical labeling used mouse monoclonal antibodies against human myosin heavy chain (MHC) (slow, fast, neonatal, developmental), spectrin, dystrophin (all from Novocastra Lab. Ltd., Newcastle, United Kingdom), desmin and laminin (DAKO, Glostrup, Denmark). Briefly, non-fixed sections were incubated in the primary antibody for 1 h, washed in buffer (TBS, pH 7.6), incubated with secondary antibody for 1 h (rabbit anti-mouse, 1:200, Zymed Laboratories, San Francisco, CA, USA) followed by incubation with the ABC kit (DAKO, Glostrup, Denmark). Antibody visualization was performed using diaminobenzidine (DAB, Sigma Aldrich, St. Louis, USA).

Morphometry

Sections were analyzed under a light microscope equipped with a computer-based image analysis system (Easy Image Measure Module 2000, Bergstrom Instrument AB, Stockholm, Sweden) to calculate area using semi-automated tracing or hand digitizing minor fiber diameter. Qualitative measures such as percent central nuclei, percent angular/abnormal fibers, percent necrotic fibers, percent MHC hybrids and percentage of each MHC isoform were judged by a single observer. Hybrid fibers were defined as those fibers with positive staining for both slow and fast MHC isoform. To estimate the amount of collagen in the section, Sir Red staining was used. Normal muscle contains primarily types I and III collagen [12]. Tissue vascularity was calculated using the α-laminin antibody which binds to both the muscle fiber basement membrane and endothelial basement membrane enabling easily visualization.

Based on our previous study, we used standard stereological principles to determine the number of fiber needed to obtain an accurate estimate of fiber area for that biopsy [26]. Using the relationship between fiber area variance and the number of fibers counted, the total number of fields quantified from each collection of images was determined to be five to six. Thus, six images were randomly selected from images taken at random from the sections, with the use of a random number generator, for cross-sectional area analysis. In cases where there were fewer than six images obtained, all images from that region were analyzed.

Statistics

Data were screened for normality and skew to justify the use of parametric tests (Kolmogorov-Smirnov). Means were compared between No BTX and BTX groups by one-way analysis of variance (ANOVA). The relationship between fiber area and age was evaluated in two different ways (based on the fact that age variable was continuous but nonnormally distributed). Data were first analyzed by simple linear regression to define the relationship between fiber size and other age between groups. A second analysis was used in which age was binned into three groups: young (age $\leq$ 7), middle (7 < age $\leq$ 14) and old (14 < age). Fiber size was compared across groups (age and size as grouping factors) using two-way ANOVA. As an extension of the ANOVA analysis, to account for the fact that the BTX group was older than the No BTX group (and older fibers are typically larger fibers), analysis of covariance (ANCOVA) was used to compare adjusted means between groups, using age as the covariate. Numerous morphometric parameters

were obtained from sections, 23 in all. Many of them covary (e.g., fast fiber size with fiber size). Thus, a correlation matrix was generated, and stepwise regression performed to define the best predictor of fiber size. Any continuous variable was allowed to enter the model with F-to-enter = 4.000 and F-to-remove = 3.996. P-values < 0.05 were considered statistically significant. Data are presented in the text as means ± SEM except where indicated.

RESULTS

A total of 25 biopsies were collected from 6 different skeletal muscles. Six fields of view that were free of freezing or sectioning artifact were used for fiber area calculation In three of the biopsies, only four fields of view were obtainable. Before analysis, each image was inspected, and areas with sectioning artifacts, blood vessels, merged fibers, or poor staining quality were omitted from the quantification. Acceptable sections had even staining, were free of freezing artifact, had tightly packed fibers and no obvious tears (Figure 1A) and all antibodies showed excellent selective labeling of individual fibers (Figure 1B).

Regression of age on fiber diameter revealed a significant positive relationship for the BTX

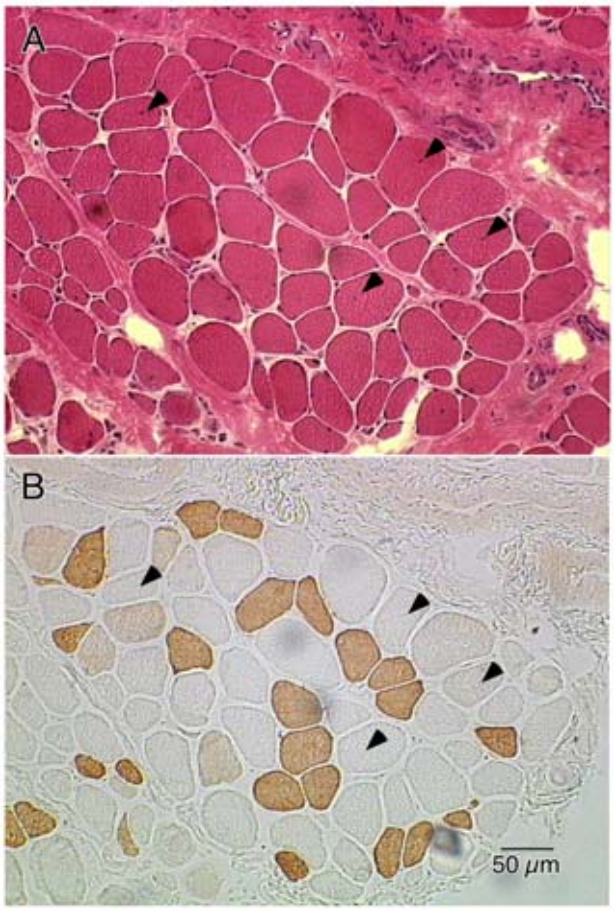

Figure 1. Serial sections of flexor carpi ulnaris muscle from a 17 year-old subject with tetraplegic cerebral palsy without BTX treatment. **(A)** Hematoxylin and Eosin staining shows relatively normal muscle morphology with slightly increased perimysial tissue and central nuclei (arrows), indicative of fiber regeneration. **(B)** Anti-slow MHC immuno-labeled tissue reveals a gradient in staining for slow MHC. Most fibers with a faint, background stain for slow MHC were also positive for fast MHC, indicating a hybrid fiber. Arrows in (B) mark the same fibers as (A) with central nuclei. Calibration bar applies to both micrographs.

(r^2=0.36; p<0.02) and for the No BTX group (r^2=0.37; p<0.03, Figure 2A). Similar results were observed on fiber area although the residual errors were larger for area than diameter and thus diameter was used for multivariate analyses. Average muscle fiber diameter and muscle fiber area were both significantly greater in muscles from BTX-treated subjects compared to subjects who had No BTX treatment (p<0.01, Figure 2B, Figure 3A). This significant difference was true for both fast and slow muscle fibers (p<0.05; data not shown). Since the age range differed slightly between BTX and No BTX groups (Table 1), we compared both mean fiber diameter and fiber area, using age as a covariate. This analysis yielded the same result, a significant difference in fiber size between groups, even after correcting for differences in age range (p<0.001, data not shown).

Skeletal muscles from the BTX-treatment group showed a greater fraction of pathological signs compared to the No BTX group. For example, while the No BTX muscles had a 'normal' [27, 28] central nuclei percentage of 1.44 ± 0.35%, there was a significant three-fold increase (5.26 ± 1.1%) of central nuclei in BTX-injected muscles (p<0.01, Figure 3D). This result indicates that the injection process induced a degeneration-regeneration cycle that was not simply a consequence of chronic spasticity. This abnormality is consistent with the fact that the injected muscles showed many other pathological signs including an increased fraction of angular fibers (p<0.05, Figure 3C), increased percentage of fibers expressing neonatal MHC (p<0.01), and an increased percentage of fibers expressing hybrid fibers (p<0.05, Figure 3B). BTX-injected muscles also demonstrated significantly increased capillarity compared to uninjected muscles. The number of capillaries per fiber (C/F) as well number of capillaries around a fiber (Figures 3E and 3F; CAF; which normalizes for fiber size differences) in the injected muscles was about 50% greater that of the non-injected muscles (p<0.05). The remainder of the parameters revealed no difference between treatment groups (data not shown). For example, no significant difference in fiber type percentage, percentage necrotic fibers or percentage ECM was observed between groups (p>0.4) and all absolute values were within normal limits [13]. Spectrin and desmin staining was localized normally and, as expected, in biopsies with many small regenerating fibers, desmin staining was consistently strong (data not shown).

Since 17 parameters were measured from each biopsy, many of which covaried with one another, stepwise regression was used to predict the parameter(s) that had the greatest effect on fiber size (Figure 4A). Surprisingly, only two parameters entered the stepwise regression model, capillary/fiber ratio (r^2=0.75, p<0.001) and capillary/area

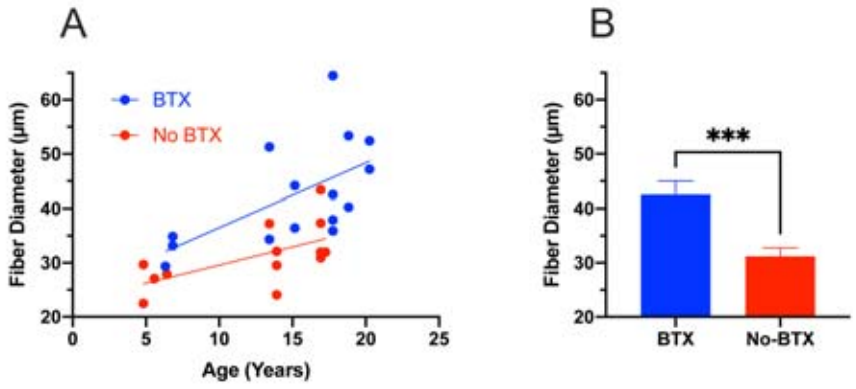

Figure 2. (A) Relationship between age and fiber diameter of No BTX-treated patients (blue) and BTX-treated patients (red). Linear regression revealed a significant relationship between age and diameter for both BTX-treated patients (p<0.05, r^2=0.36) and No BTX-treated patients (p<0.05, r^2=0.37). While the slope was almost twice as great for the BTX group compared to the No BTX group (1.29 µm/year vs. 0.67 µm/year), these differences were not significant (p>0.3). **(B)** Mean fiber diameter compared between groups, averaged across all ages. The BTX group had a significantly larger mean diameter compared to the no BTX group (p<0.001, indicated by asterisks).

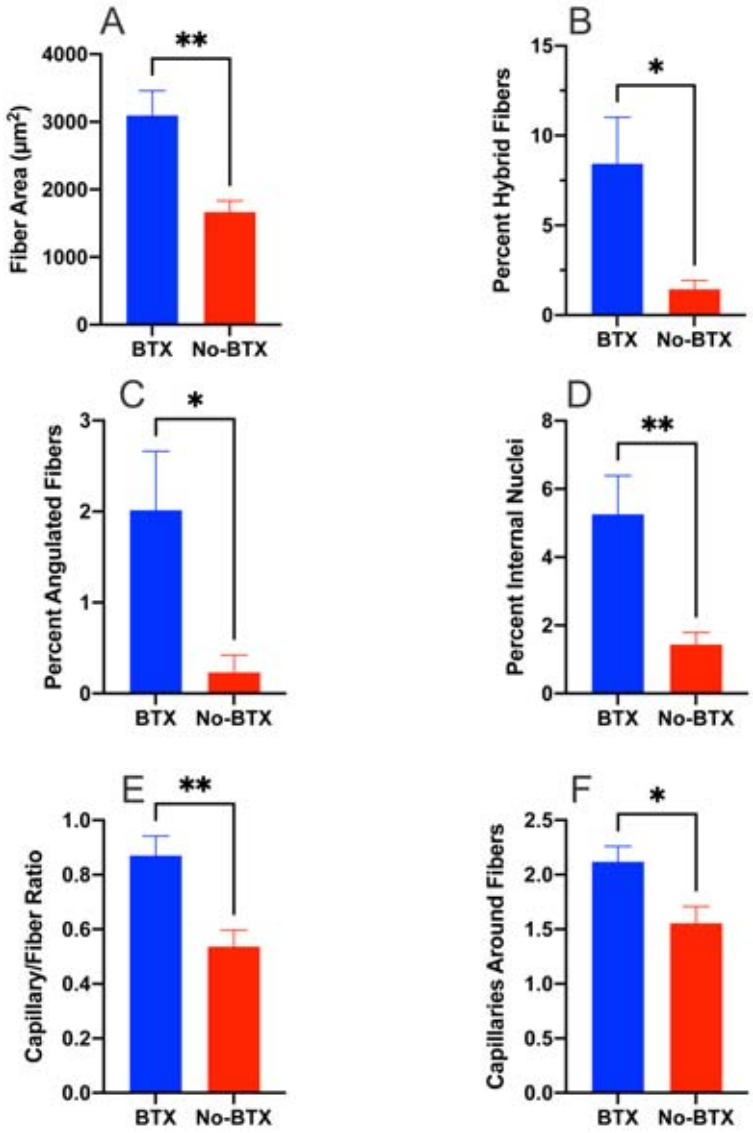

Figure 3. Morphometric parameters measured for BTX and No BTX experimental groups. **(A)** Muscle fiber area**. (B)** Percent hybrid fibers expressing both fast and slow myosin heavy chain. **(C)** Percent angulated fibers. **(D)** Percentage of fibers with internal nuclei, suggesting fiber regeneration, **(E)** Capillary-to-fiber ratio. **(F)** Number of capillaries around each fiber (Significant difference between groups indicated by asterisks; *, $p<0.05$; **, $p<0.01$).

(partial $r^2=0.06$, $p<0.001$), and, together, they provide an excellent, linear prediction of fiber diameter (Figure 4B; $r^2=0.81$, $p<0.0001$). Importantly, in spite of the correlation between age and fiber size, age was never included as a factor suggesting that age alone did not determine fiber size.

DISCUSSION

The purpose of this study was to define the morphometric properties of spastic human skeletal muscle that had been injected with Botulinum toxin type A (Botox®; BTX) compared to non-injected spastic muscle (No BTX). Spastic human muscle tissue from contractures demonstrates a number of abnormalities never seen in animal models such as hyperelongated sarcomeres [11, 29], unique transcriptional profiles both in upper [30] and lower extremity muscles [31] that are distinct from simple immobilization-induced atrophy [32] and the degeneration-regeneration

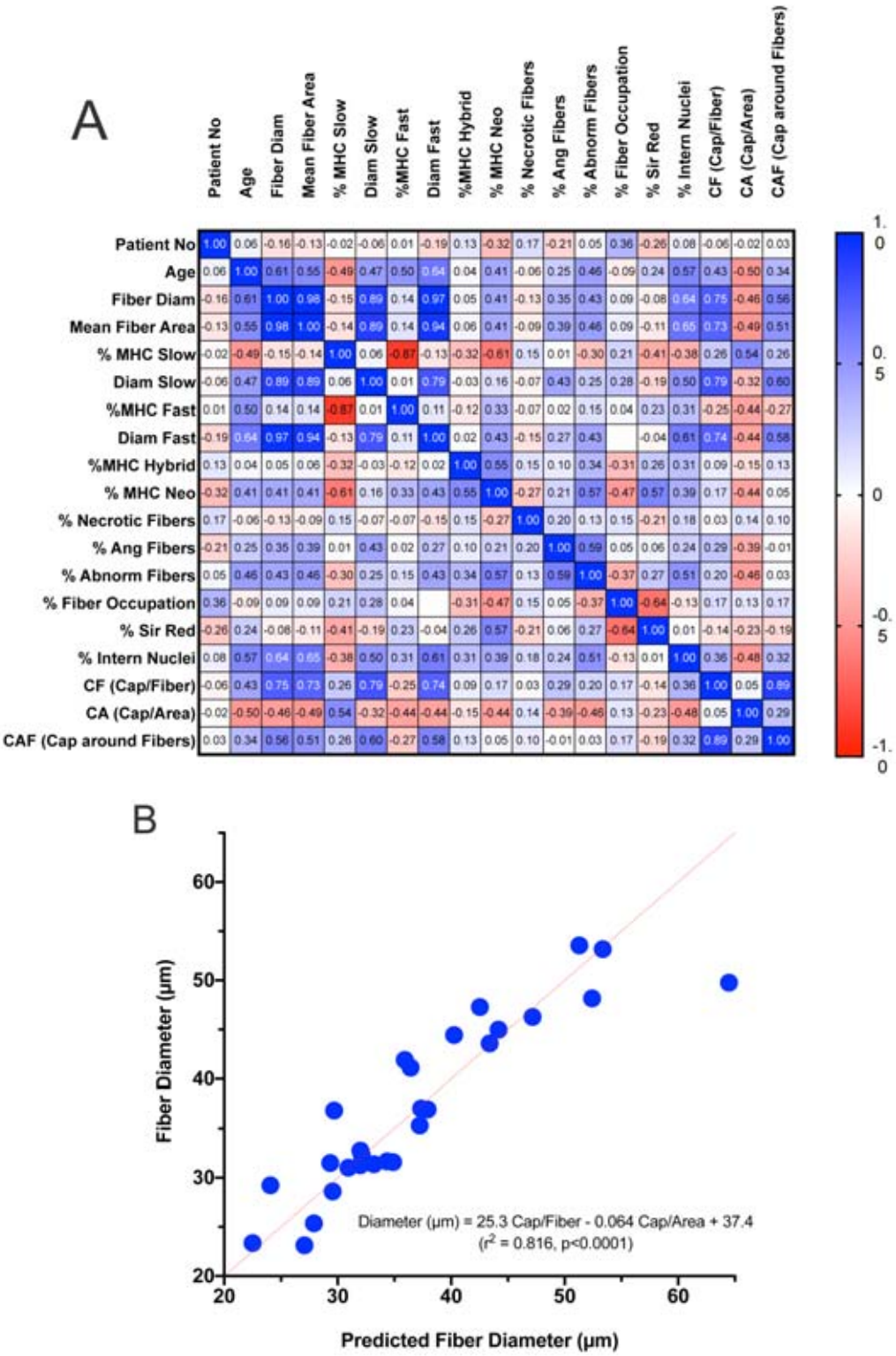

Figure 4. Multivariate analysis of morphometric data. **(A)** Correlation matrix across all 17 parameters measured and patient age. Note that all areas and diameters are highly correlated as would be expected. **(B)** Stepwise regression of all parameters (except areas) to predict measured fiber diameter. Note only two parameters, capillaries/fiber and capillaries/area entered the regression equation which was significant ($p<0.0001$) and highly linear ($r^2=0.82$).

cycles that are typical of Duchenne Muscular Dystrophy [33, 34]. Thus, we suggest that spasticity and the resulting contracture is a uniquely human phenomenon and results from abnormal input created by the central nervous system. If this is true, it may not be surprising that BTX injection resulted in the counterintuitive result presented above, very distinct from previous results in animal models.

Importantly, we found pathological signs in injected muscles that imply these muscles had undergone degeneration-regeneration. This was not simply a secondary result of spasticity as non-injected 'control' subjects with CP did not exhibit this change. Even more interesting is that these pathological signs were observed such a long time after BTX administration (from 6 months to 4 years) indicating a long-lasting process. While these pathological findings were highly significant statistically, the actual magnitude of the pathology (Figure 3) was still very low. However, perhaps more important, we also found that the injected muscle cells were larger compared to the non-injected muscles. Since muscle fiber size is typically correlated with strength, the intrinsic force-producing ability of BTX injected muscles may be higher than the No BTX group. It must be stated that increasing the strength of a muscle is never the motivation for the use of a neurotoxin. However, in both of our transcriptional analyses [30, 31], an explicit comparison of fiber size between children with CP and typically developing children (see Figure 4 of [35]), and a detailed measurement of whole muscle volumes by MRI [36], it is clear that muscles of children with CP have smaller fibers and smaller volumes compared to their typically developing peers. Additionally, the transcriptional profiles suggest an increase in the myostatin signaling apparatus that may itself suppress growth [30, 31]. We posit that the upper motor neuron lesion associated with CP exerts an active anti-trophic or at least a growth-suppressing influence, perhaps *via* the neuromuscular junction. If this is the case, perhaps it can explain the larger fiber size observed in this study—BTX 'inhibits' the 'growth inhibition' influence of the upper motor neuron lesion allowing more growth. It is also possible that BTX injection permits an altered use pattern that ends up being beneficial to the muscle tissue. Clearly, the growth is not normal and muscle fibers of children with CP are smaller than their typically developing counterparts. This is seen in the slope of the fiber size vs. age relationship, which in this study is approximately 100 μm^2/year (Figure 2) compared to the typically developing value of about 250 μm^2/year (Figure 4 of [35]).

We were surprised that the best predictor of fiber size (either area or diameter as shown by stepwise regression) was C/F ratio. This was not due to a large shift in fiber type that might accompany a massive regeneration response since slow fiber percentage was ~45% in both experimental groups (data not shown) Stepwise regression demonstrated a very strong preference for C/F as a predictor of fiber size since this value explained over 80% of the fiber size data variability This is not surprising since C/F ratio is typically proportional to fiber diameter [37] and increased capillarity is characteristic of regenerating muscle [38]. This may indicate a strong regenerative impact of the BTX. While the C/F and CAF values were good predictors, they were substantially lower than that which has previously been reported for healthy subjects [37] indicating a lower vascularization in spastic skeletal muscle [39].

How does one interpret these 'mixed' results? The 'positive' aspect of BTX injection (*i.e.,* increased fiber size) must be weighed against the 'negative' pathological signs observed in the tissue, namely, increased percentage of central nuclei, angular fibers and fibers expressing neonatal MHC isoforms. Whether hybrid MHC fibers, expressing both fast and slow isoforms, should be considered pathological, is debatable [40, 41]. However, it is clear that the injected muscles show greater pathological signs compared to non-injected muscles (Figure 3). The pathological signs considered here are established signs of skeletal muscle regeneration, features known to occur after temporal denervation caused by neurotoxic injections into skeletal muscles. However, the regeneration seen in the biopsies from injected muscles, taken from 5 months to 4 years after the BTX administration, certainly indicates a far longer lasting activity than one would suspect. Several studies in animal models have not

reported any vigorous regenerative response of muscle after neurotoxin injection [22, 42, 43] but it must be emphasized that these animal models involve normal, not spastic skeletal muscle.

There is precedent in the skeletal muscle plasticity literature that, by inducing a muscle regenerative response, tissue plasticity increases [44]. Thus, regeneration is used routinely in studies of muscle development to recapitulate the developmental process by activating intramuscular sources of stem cells [45, 46]. This raises the provocative possibility that the induction of regeneration secondary to neurotoxin is actually beneficial to the muscle tissue by activating endogenous sources of myogenic potential. Additional manifestation of ongoing regeneration was the higher incidence of small fibers expressing neonatal MHC in BTX treated skeletal muscle. While the percentage of ECM was higher than expected for normal skeletal muscle (consistent with the animal studies in [47]), there were no differences between groups indicating that BTX injection did not inhibit fibrosis development in spastic skeletal muscle tissue. Thus, one cannot rule out the possibility that the pathological signs found in this study are due to a failure in regeneration processes eventually leading to an increased fibrotic skeletal muscle tissue.

CONCLUSION

In summary, this study provides evidence that could be considered either 'positive' or 'negative' regarding the use of BTX depending on one's interpretation of the classic muscle physiology and histology literature: Based on fiber size alone, one would conclude that the BTX-injected muscles were stronger compared to the non-injected muscles and that they had grown more readily. However, based on the increased pathological signs, one would conclude that the BTX-injected muscles had a prolonged and/or disturbed regeneration. The relative balance and reasons of these effects in patient population remains the key question in terms of applying these results to clinical practice.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grants AR40539 and HD44822, the United Cerebral Palsy Foundation, and the Department of Veterans Affairs. The authors acknowledge the contribution of Eva Runnesson, Ph.D., who contributed to this work but was unable to be found to read the manuscript and serve as an author. The authors would like to thank Dr. Hank Chambers for helpful comments on this work and providing surgical samples. Dr. Lieber was partially supported by the Fulbright Foundation as a Fellow to Sweden in 2007 under a program administered by the International Council for the Exchange of Scholars (ICES, Washington, D.C.).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest regarding this study.

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