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Effects of Repeated Eyelid Injections with Botulinum Toxin A on Innervation of Treated Muscles in Patients with Blepharospasm

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Abstract

Purpose: To assess changes in innervation and muscle morphology after repeated botulinum toxin A injections in subjects with benign essential blepharospasm.

Methods: Surgical waste specimens were processed for histologic examination of nerve fibers, neuromuscular junctions, fiber size, and central nucleation and compared to age matched controls and to two subjects with blepharospasm that had not received botulinum toxin A injections

Results: There was a significant increase in amount of nerve fibers and numbers of neuromuscular junctions in the orbicularis oculi muscles from subjects with blepharospasm treated repetitively with botulinum toxin A. In addition there was a significant decrease in mean muscle fiber cross-sectional area and an increase in central nucleation. The specimens from the subjects with only blepharospasm had the same density of nerves but had intermediate levels of neuromuscular junctions.

Conclusions: These data suggest that repeated injections of botulinum toxin A has an effect on nerve and neuromuscular junction numbers, which are partly mirrored in orbicularis oculi muscle from subjects with blepharospasm only. These studies suggest the potential for modulating these changes in order to extend the duration of effectiveness of botulinum toxin.

Précis:

Repeated botulinum toxin A injections in blepharospasm subjects' orbicularis oculi resulted in smaller muscle fibers, central nucleation, and increased nerve and neuromuscular junctions.

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Keywords

botulinum toxin A; orbicularis oculi muscle; blepharospasm; peripheral nerves; neuromuscular junctions

INTRODUCTION

Benign essential blepharospasm is a form of focal dystonia,[1] with an incidence rate of 1.2 persons per 100,000 per year[2] and prevalence rates from 16-133 per million in multiple studies.[3] Prevalence estimates for all forms of primary dystonia range from 2-50 cases per million of the population for early onset and 30-7320 cases per million for late-onset dystonia.[4] Botulinum toxin A, which blocks the release of acetylcholine at the neuromuscular junction, resulting in paralysis, was originally developed as a potential treatment for strabismus.[5] Its utility in the management of blepharospasm was realized shortly thereafter.[6] Since these initial studies, the intramuscular injection of botulinum toxin A has safely relieved dystonia symptoms for thousands of patients of all ages[7] as well as significantly improved their quality of life.[8,9]

Research into the mechanism of action revealed the botulinum toxin A acts specifically on the presynaptic terminal, where it cleaves the protein SNAP25.[10] The effect of botulinum toxin persists for several months as the cleaved SNAP25 is retained at the neuromuscular junction, preventing the insertion of new SNAP25 into the nerve terminal.[11] However, function slowly returns after botulinum toxin-induced muscle paralysis. A number of studies have demonstrated nerve terminal and nodal sprouting in the paralyzed nerves as early as two days after botulinum toxin injection.[12,13] Evidence of axon sprouting after botulinum toxin A injections was seen specifically in human orbicularis oculi muscle from blepharospasm subjects at the single neuromuscular junction level.[14,15] This axon sprouting was postulated to be responsible for the formation of de novo neuromuscular junctions over the orbicularis oculi muscle surface, increasing the speed of the return of muscle function . Using rabbits, an injection of botulinum toxin A resulted in a significant increase in neuromuscular junctions throughout the paralyzed orbicularis oculi muscles,[16] with a similar finding seen after injection of botulinum toxin A into rabbit extraocular muscles.[17]

Individuals with benign essential blepharospasm and other focal dystonias will receive many series of botulinum toxin A injections. The potential effects of repeated injections on the orbicularis oculi muscle and its innervation are not known. In a study of long term efficacy, there was a significant increase in botulinum toxin A dose between the first year and last year of treatment (with a range of 10-20 years of treatment), but no significant difference in the duration of effect.[18] The current study focused on the potential effects of repeated botulinum toxin A injections on muscle and nerve density using surgical waste specimens from subjects who underwent an orbicularis oculi myectomy. These were compared to naïve orbicularis oculi specimens removed during blepharoplasty in the absence of focal dystonia in those subjects.

METHODS

All studies were approved by the Institutional Review Board at the University of Minnesota, de-identified, and subsequently processed without knowledge of the source or type of specimen. Table 1 gives the age, sex, and number of botulinum toxin A injections per individual and dose, the age of the two specimens from a subject with benign essential blepharospasm who never received an injection of botulinum toxin, and five age-matched control individuals who underwent an upper lid blepharoplasty in the absence of focal dystonia or other neuromuscular disorder. At the time of regularly scheduled orbicularis oculi myectomy surgery (performed by ARH or AM), the pretarsal and preseptal muscles were excised separately and were placed in physiological saline on ice. Within 1-3 hours, they were embedded in tragacanth gum and frozen in 2-methylbutanc chilled on liquid nitrogen. The tissue blocks were stored in a −80°C freezer until sectioned.

The muscle specimens were sectioned at 12 μm using a cryostat. Representative sections through the entire thickness were stained using hematoxylin and eosin by standard methods. A series of at least 3 sections were immunostained using an antibody to neurofilament protein (smi-31; 1:30,000; BioLegend, San Diego, CA) to identify nerve fibers. Sections were incubated in normal serum at 10% in 1M phosphate buffered saline (PBS) containing 0.01% Triton X-100 (antibody buffer), incubated in antibody buffer overnight at 4°C in humid chambers, rinses in PBS, and the incubated in secondary antibody using the Vectastain Elite kit (Vector Laboratories, Burlingame, CA) following package instructions. The sections were then rinsed in PBS, and reacted with diaminobenzidine and 0.1% hydrogen peroxide, using heavy metals to intensify the reaction. Slides were rinsed in PBS, dehydrated in graded alcohols, and coverslipped with Permount (Fisher Scientific, Pittsburgh, PA). A second series of three slides per tissue block were stained for the visualization of neuromuscular junctions. Slides were rinsed in PBS, and incubated with α-bungarotoxin conjugated to Alexa Fluor 488 overnight in a humid chamber at 4°C. Following a PBS rinse, the sections were coverslipped using Vectashield mounting medium (Vector Labs.).

Morphometric analyses were performed. Mean cross-sectional areas and percent of myofibers with central nuclei, indicative of degeneration/regeneration or denervation/reinnervation, were measured manually using Bioquant Nova Prime software (Bioquant, Nashville, TN). A minimum of 3 slides were analyzed, and a minimum of 200 fibers were examined per muscle. For any given subject, the right and left sides of the same region (e.g. pretarsal) were averaged, and the averages were used to determine the means for the botulinum toxin A treated muscles from subjects with benign essential blepharospasm and for the naïve control muscles. For the two subjects with benign essential blepharospasm who were never treated with botulinum toxin A, the average of the left and right sides in each region were calculated, and the means from the 2 muscles were then averaged. For nerve fiber analysis, a minimum of 3 slides were analyzed from each muscle region from both right and left eyelids. For each muscle from each eyelid area (pretarsal and preseptal), all the smi-31-positive nerve fibers within any given microscopic field of view were manually circled as was the total area of the field. The entire section was measured, and percent area containing nerve fibers was determined and compared to muscle area and compared to tissue

area (muscle plus connective tissue). Similarly, every neuromuscular junction was counted in each of the three muscle sections, and the entire tissue section area was measured, to determine number of neuromuscular junctions relative to tissue area. As for the fiber areas, specimens from the same muscles from each individual were averaged to determine the overall averages.

The mean ± SEM were reported. Data analyses of the blepharospasm and botulinum toxin treated measurements compared to naïve controls used an unpaired Students' t-test and Prism software (Graphpad, San Diego, CA). Statistical significance was defined as $P<0.05$.

RESULTS

The mean age of the control subjects was 64.8 (range: 59-69), the mean age of the subjects with blepharospasm who were treated with repeated botulinum toxin injections was 61.5 (range: 55-68), and the mean age of the subjects with blepharospasm only was 59.5 years (Table 1). Thus the collected specimens were closely age-matched. Table 1 gives the number of botulinum toxin injections and doses (if known) for each of the treated subjects, with a mean of 16.8 injections (range 3-28) per botulinum toxin treated subject.

When the amount of nerve fibers is calculated based on percent of total cross-section (Figure 1A) or percent of muscle cross-sectional area (Figure 1B), the blepharospasm and botulinum toxin A- treated specimens had significantly more nerve fibers than naïve control muscles, showing differences of 48.1% and 47.3%, respectively. While statistical analysis could not include the muscle specimens from the two subjects with blepharospasm who had not been injected with botulinum toxin A, these specimens had 34.7% more nerve as a percent of total tissue in cross section and 11.1% more nerve as a percent of muscle tissue in cross-section. These values are 14.2% and 36.7% less than the nerve densities from the blepharospasm and botulinum toxin A- treated specimens.

This analysis was extended to analyze each muscle region separately. In the pretarsal region, there was significantly more nerve tissue both as a percent of all tissue and as a percent of muscle tissue, 87.8% and 102.2% respectively (Figure 2). In the preseptal region, the blepharospasm and botulinum toxin treated muscles had 44.5% and 31.1% more nerve fibers than the naïve control tissues, but only the nerve per total cross-section was statistically different. It is interesting to note that in both the pretarsal and preseptal regions of the orbicularis oculi muscle from the subjects with blepharospasm but without botulinum toxin A injections, the nerve density as a percent of either total cross-section or muscle cross-section was similar to the muscles from the subjects with both blepharospasm and botulinum toxin A treatment.

In order to assess changes on the postsynaptic side, neuromuscular junctions were localized using α-bungarotoxin staining and quantified morphometrically (Figure 3). There were significant increases in neuromuscular junctions per mm^2 in both the pretarsal and preseptal regions of the muscles from subjects that had blepharospasm and botulinum toxin A treatment compared to the age-matched control muscles from the same muscle regions, with a 5-fold and a 4-fold difference respectively. In contrast to the nerve density measurements,

the pretarsal muscles from the blepharospasm subjects untreated with botulinum toxin A showed a level of neuromuscular density approximately equal to that of the control muscles, but 4-fold fewer than that of the blepharospasm muscles treated with repetitive botulinum toxin A injections. In the preseptal region, there were relatively similar levels of neuromuscular junctions in the control and blepharospasm only muscles; however there was a 114.7% difference between the controls and the botulinum toxin A treated muscles and an 81.5% difference between the treated and blepharospasm only muscles. This suggests that neuromuscular junction changes are increased with repetitive botulinum toxin A injections. It should also be noted that while there were many more neuromuscular junctions in the muscles from the botulinum toxin A-treated blepharospasm subjects, they tended to be smaller and thinner (Figure 3A, B).

The mean cross-sectional area of the pretarsal region of the muscle specimens from the subjects with benign essential blepharospasm treated with multiple injections of botulinum toxin A were significantly smaller than the untreated control muscles, with a difference of 24.8% (Figure 4A-C). The myofibers in the preseptal region were also significantly smaller, with a difference of 52.4% in the botulinum toxin A-treated muscles from blepharospasm subjects compared to controls (Figure 4). It is also interesting to note that the muscles from the subjects with only blepharospasm had similar areas to the control muscles, with a 7% and 6.5% difference respectively. Central nucleation is a hallmark sign of degeneration/regeneration or denervation/reinnervation. In both the pretarsal and preseptal regions, there was a significant increase in percent of myofibers with central nucleation in the botulinum toxin A-treated muscles from subjects with blepharospasm, with a 6-fold (129.8%) and a 2-fold (111%) increase respectively (Figure 4A,B,D). The muscles from the subjects with only blepharospasm had intermediate central nucleation levels, 72.5% and 71.6% greater respectively.

DISCUSSION

Previous studies examining individual neuromuscular junctions in subjects with blepharospasm and repeated botulinum toxin A injections showed marked evidence of terminal sprouting in the excised orbicularis oculi muscles.[14,15] In the present study, we showed that multiple injections of botulinum toxin A over time resulted in a number of widespread changes to the orbicularis oculi muscles of these subjects with blepharospasm. Botulinum toxin A injections caused a muscle wide increase in both nerve fiber and neuromuscular junction density compared to naïve control orbicularis oculi muscles. While statistics cannot be reliably performed on four specimens from two subjects with blepharospasm and no botulinum toxin A treatments, it is interesting that only the nerve densities were similar to the botulinum toxin A treated orbicularis oculi muscles with neuromuscular junction levels the same as the control muscles. This suggests that the increased number of nerve fibers per tissue area may be specifically related to the blepharospasm and not due to up-regulation by the repeated botulinum toxin injections. As there are only orbicularis muscles from two untreated blepharospasm subjects, the data must be interpreted carefully; It is, however, quite interesting given a condition whose primary etiology is not understood. This does not negate the literature which clearly has demonstrated that botulinum toxin A-induced nerve paralysis results in nerve sprouting in

the area of the chemically denervated muscle fibers.[12,19] This is important to note that this sprouting occurs in the absence of physical damage to the nerve fibers, as physical damage is known to induce significant nerve fiber sprouting from the end of the injured nerve.[20]

The increase of *de novo* neuromuscular junctions as a response to muscle paralysis with botulinum toxin has been described in a number of muscles,[17,21–23] and specifically in the orbicularis oculi muscles.[15,16] These extrajunctional acetylcholine receptors also form in response to nerve section.[22] As we demonstrated after botulinum toxin injection into the extraocular muscles, the induced muscle paralysis results in significant increases in neuromuscular junctions throughout the length of the muscles.[17] There are strong similarities between the response to botulinum toxin A injection in the orbicularis oculi muscles and extraocular muscles. Both sets of muscles have short myofibers that do not span the length of the muscles,[17,24,25] have more than one neuromuscular junction on single muscle fibers,[24,26] and have small mean cross-sectional areas compared to limb skeletal muscles.[27–29] It is interesting that there was not as large an increase in neuromuscular junction density in the specimens with blepharospasm only. While one must be careful not to over-interpret data from two subjects (4 muscles), these data suggest that while chemodenervation causes a large increase in neuromuscular junction number across each specimen, a good proportion of this response is due specifically to the botulinum toxin treatment. This observation, and the elevation in the number of nerves in the specimens with blepharospasm only, makes it tempting to speculate that at least part of the peripheral manifestation of blepharospasm might be due to some level of denervation and subsequent reinnervation. These specimens are quite rare, as the general standard of care for blepharospasm is botulinum toxin injections. Examination of additional specimens of orbicularis oculi muscle from blepharospasm patients who have not undergone treatment with botulinum toxins will allow us to better understand the pathophysiology of this disorder.

In peripheral skeletal muscles, the almost universal response to botulinum toxin injection is temporary muscle atrophy.[30–32] This was even seen in leg muscles after the larger doses of botulinum toxin A needed for the treatment of cervical dystonia.[33] Botulinum toxin A also produced muscle atrophy in the masseter,[34] and has been used to reduce masseter muscle hypertrophy in human subjects.[35] Similar types of muscle fiber atrophic changes were described in experimental studies of the effects of botulinum toxin A into orbicularis oculi including human subjects.[36,37] In a previous study using human surgical waste tissues from blepharospasm subjects treated with botulinum injections, extreme variability in myofiber cross-sectional areas was noted.[38] Relative to these studies, we saw a significantly decreased mean myofiber cross-sectional area only in the preseptal portion of the treated orbicularis oculi muscle compared to naïve control. However, it is important to note that the muscle specimens from the subjects with blepharospasm only did not show a decrease in myofiber cross-sectional area. Thus one can speculate that these changes are likely due to the repeated exposure to botulinum toxin A.

The studies thus far have shown that the innervation to and structure of mammalian neuromuscular junctions have a fair bit of plasticity,[12,17,19,21–23,39] and these features are altered for long durations after botulinum toxin injection. This plasticity has the potential to

be modified by other treatments as well. A number of studies have shown that the terminal nerve sprouting in denervated skeletal muscle can be altered in multiple ways, for example by changing neurotrophic factor levels, such as insulin-like growth factor (IGF-1),[40] by modifying levels of other molecules such as corticotrophin releasing factor (CRF),[16] and by injection of blocking antibodies for neural cell adhesion molecule (NCAM),[41,42] tenascin,[43] or IGF-1.[16] These studies suggest the potential for these types of approaches to extend the duration of effectiveness of botulinum toxin. The orbicularis oculi muscle specimens from the subjects with previously untreated blepharospasm also had increased amounts of peripheral nerve within the muscles compared to naïve, age-matched control muscles, suggesting that these peripheral changes may be part of the disease process. If borne out by future studies, these data suggest that reduction of nerve and neuromuscular junction numbers by use of CRF or blocking peptides or antibodies has the potential to be a primary treatment for blepharospasm.

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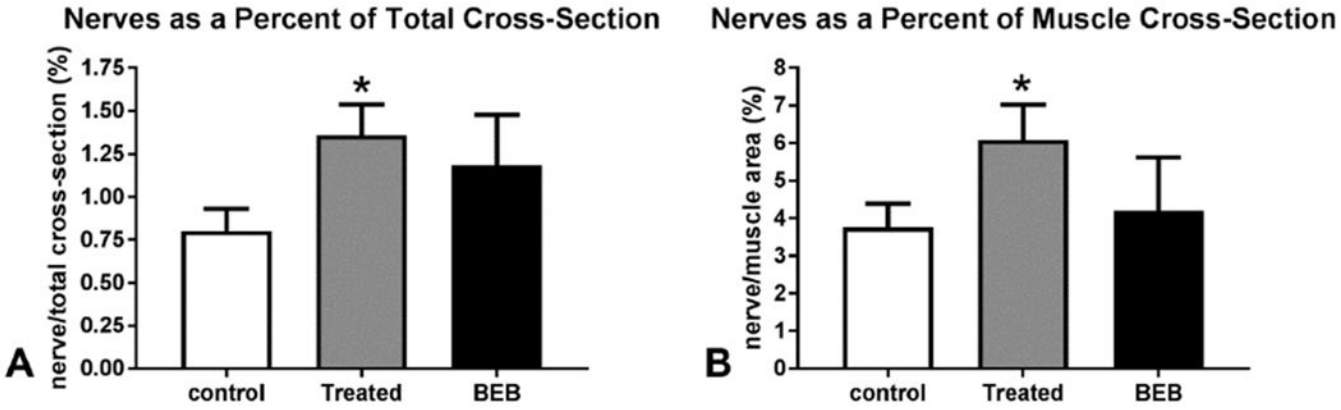

Figure 1:

Morphometric analysis of nerve fibers in surgically excised orbicularis oculi muscle as a percent of total tissue cross-section (A) or muscle cross-section (B). * indicates significant difference from control. Control: naïve control orbicularis oculi muscles; Treated: muscles from subjects with blepharospasm who had been treated with multiple botulinum toxin A injections; BEB: muscles from subjects with blepharospasm who never had an injection of botulinum toxin A.

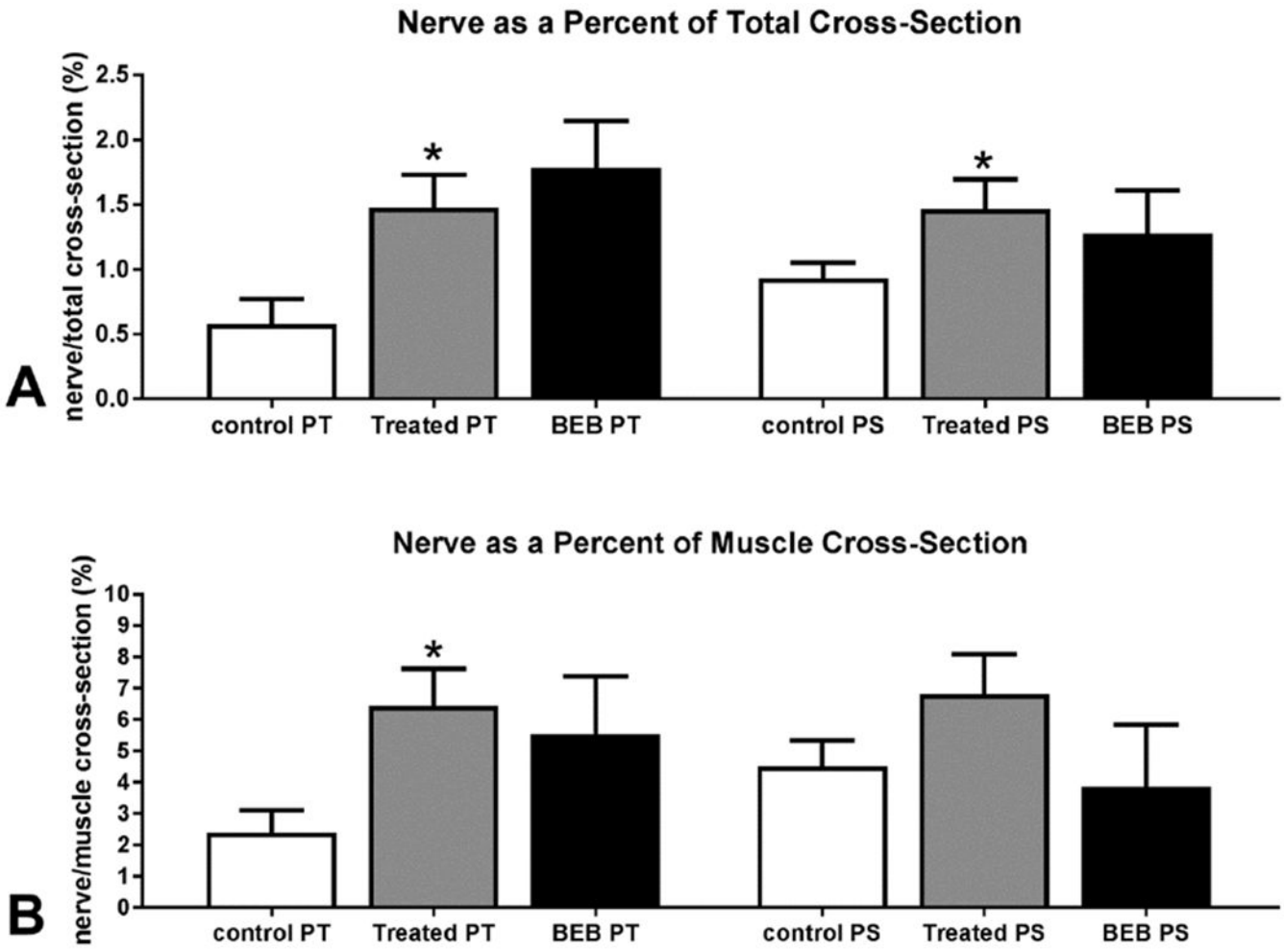

Figure 2:

Morphometric analysis of nerve fibers in the pretarsal (PT) region and preseptal (PS) region of the orbicularis oculi muscle as a percent of total tissue cross-section (A) or muscle cross-section (B). * indicates significant difference from control. Control: naïve control orbicularis oculi muscles; Treated: muscles from subjects with blepharospasm who had been treated with multiple botulinum toxin A injections; BEB: muscles from subjects with blepharospasm who never had an injection of botulinum toxin A.

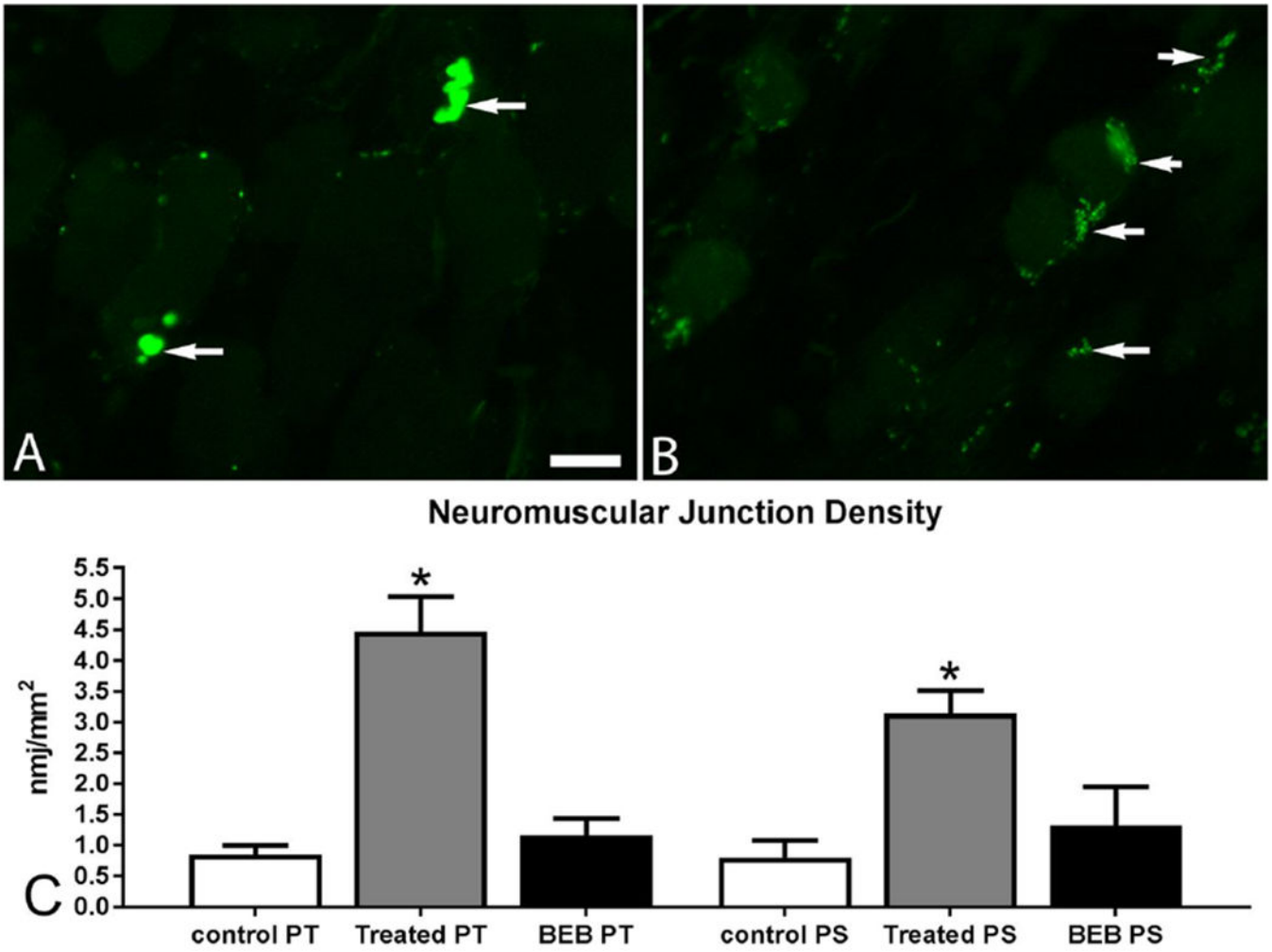

Figure 3:

Photomicrograph of neuromuscular junctions (green) stained with a-bungarotoxin conjugated to AlexaFluor 488 in (A) naïve control orbicularis oculi muscle and in (B) orbicularis oculi muscle from a subject with blepharospasm who had multiple botulinum toxin injections prior to surgical myectomy. (C) Morphometric analysis of neuromuscular junction density was a percent of mm^2 of tissue in the pretarsal (PT) and preseptal (PS) regions of the orbicularis oculi muscle from control, botulinum toxin treated muscles from blepharospasm subjects, and subjects with blepharospasm only. Bar is 30 μm. * indicates significant difference from control.

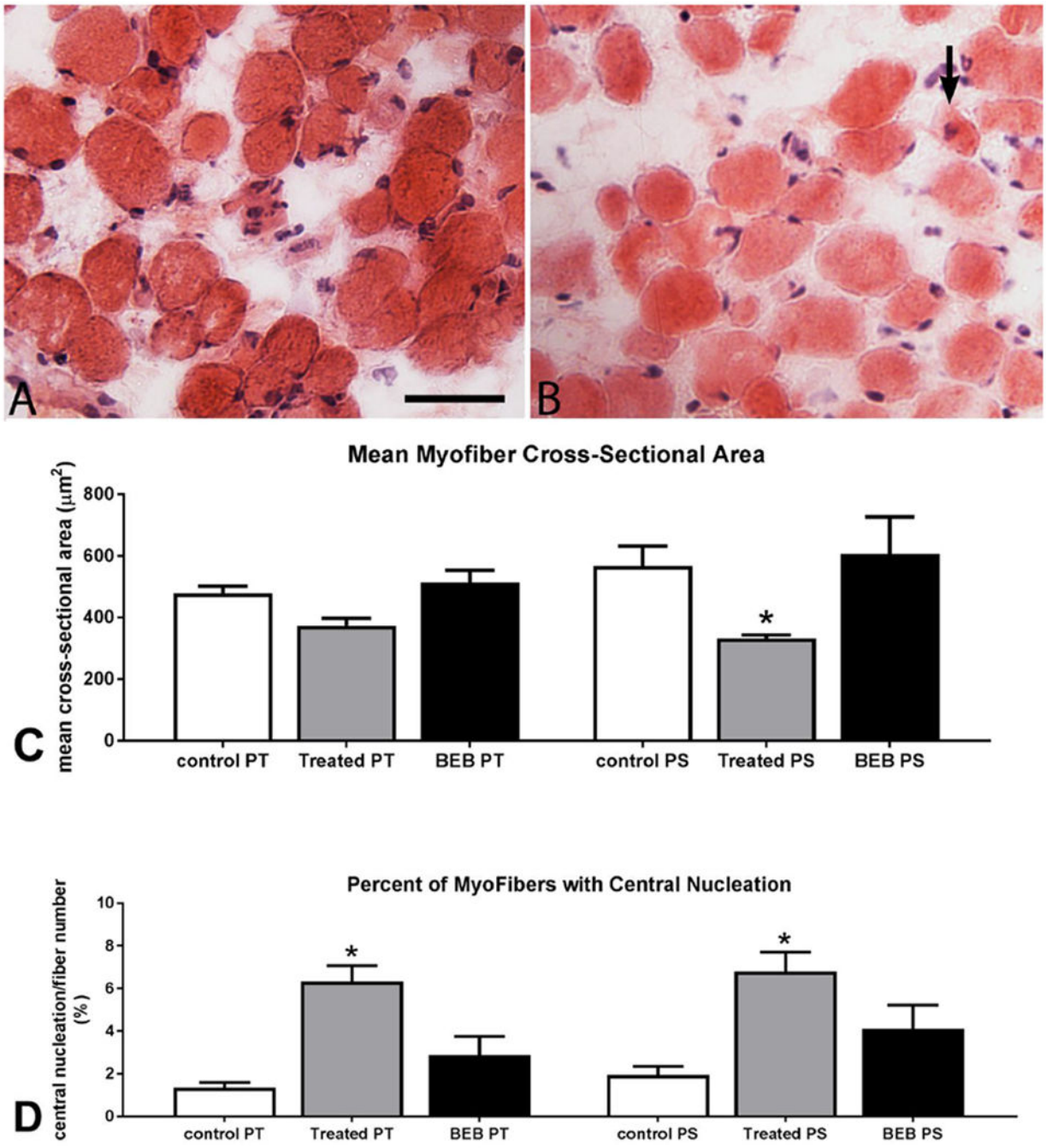

Figure 4:

Photomicrograph of (A) naïve control orbicularis oculi muscle and (B) orbicularis oculi muscle from a subject with blepharospasm who had multiple botulinum toxin injections prior to surgical myectomy. Arrow indicates a centrally located myonucleus. (C) Mean myofiber cross-sectional areas and (D) percent of myofibers with central nucleation in the pretarsal (PT) and preseptal (PS) regions of the orbicularis oculi muscle from control,

botulinum toxin treated muscles from blepharospasm subjects, and subjects with blepharospasm only. Bar is 50 μm. * indicates significant difference from control.

Table 1:

Subject Information

	Subject Age	Subject Sex	Number of Botulinum Toxin A Injections	Dose/Visit
BEB and Botox				
1	55	F	20	72.5-80 units
2	59	F	20	60 units
3	55	F	6	30 units
4	68	F	24	60-90 units
5	64	F	28	90 units
6	64	F	3	variable
BEB only				
1	59	F	None	
2	60	F	None	
Normal Controls				
1	69	F	None	
2	64	F	None	
3	64	F	None	
4	68	F	None	
5	59	M	None	