RESEARCH ARTICLE

Ultrastructural evaluation of intramuscular applied botulinum toxin type A in striated muscles of rats

Kocaelli H[1], Yaltirik M[1], Ayhan M[1], Aktar F[2], Atalay B[1], Yalcin S[1]

[1]Department of Oral and Maxillofacial Surgery, Faculty of Dentistry
[2]Department of Histology and Embryology, Faculty of Medicine
Istanbul University, Istanbul, Turkey

Abstract

Background: Botulinum toxin type A (BTX-A) is clinically utilized for therapeutic and cosmetic purposes in maxillofacial surgery as well as many other medical specialties. There is no sufficient ultrastructural research about BTX and it is controversial whether BTX-A causes muscle degeneration to some extent, in the course of therapy. The aim of this study was to evaluate the histological effects of BTX-A when injected into masseter and gluteal muscles.

Materials and methods: A total of 30 male Sprague-Dawley rats were used and randomly divided into experimental (n =15) and control groups (n =15). Masseter and gluteal muscles were injected with a single dose of BTX-A in normal saline (0.5 U/0.1 ml), or 0.1 ml of normal saline, in the experimental and control groups, respectively. After 12 weeks all the rats were sacrificed. Gluteal, masseter muscles, and the sciatic nerves of the rats were prepared and electron microscopic, and light microscopic evaluation was performed on semi-thin sections cut from Epon embedded tissues and stained with toluidine blue. Quantitative parameters such as muscle fiber thickness and qualitative assessments including sarcosomal (striated muscle mitochondria) deformation, glycogen content, features of the triad structures and the intensity of connective tissue around the muscle fibers, and endoneurial and perineural tissue around nerve fibers were evaluated microscopically. We paired BTX- A (+) and BTX-A (-) samples statistically. Independent Samples t-test was used for the statistical analysis.

Results: Muscle fiber's diameter was significantly decreased in BTX-A (+) group ($p < 0{,}001$). Atrophic changes in the myofibrils were characterized by a decrease in the myofibrillar diameter and changes in the sarcomere structure, and were prominent in the BTX-A (+) group. Also, some other changes like dilatation in the sarcoplasmic reticulum cisternae, mitochondrial swelling, and clearing of mitochondrial cristae associated with degeneration, were detected. No morphologic difference in the sciatic nerve fibers was detected, and myelin sheaths of axon structures were intact in both groups.

Conclusion: BTX-A-induced muscular changes that are predominantly related to atrophy instead of degeneration. Although predominantly related to atrophy, our degeneration related findings suggest that further studies are needed focusing on detecting BTX-A effects on a cellular level. Hippokratia 2016, 20(4): 292-298

Keywords: Botulinum toxin type A, muscular changes, myofibrils, ultrastructure, electron microscopy, atrophy, degeneration

Corresponding author: Humeyra Kocaelli, PhD, Associate Professor, Istanbul Universitesi, Dis Hekimligi Fakültesi, Agız Dis Cene Cerrahisi Anabilim Dalı, Capa (34093)/ Fatih/ Istanbul, Turkey, tel: +902124142020/30376, fax: +902125422789, e-mail: kocaelli@istanbul.edu.tr

Introduction

Botulinum toxin (BTX) is the first toxin used in medicine and is widely accepted as a medication rather than a toxin. BTX is a potent neurotoxin, produced by the bacterium *Clostridium botulinum* that exerts its paralytic effects by inhibiting the release of acetylcholine at the neuromuscular junction[1,2]. Since its introduction to clinical use over 35 years ago, it has become a versatile drug in various fields of medicine including cosmetic uses for facial wrinkles, masseter and temporalis muscle hypertrophy, therapeutic uses such as focal dystonias, vocal tics and stuttering, cricopharyngeal achalasia, various manifestations of tremor, hemifacial spasm, temporomandibular joint dysfunction, bruxism, masticatory myalgia, sialorrhea, hyperhidrosis, and headache[3-7]. Eight immunologically distinct strains of toxin have been identified and labeled as A, B, C-1, C-2, D, E, F, and G. BTX type-A, (BTX-A) is the most widely used BTX preparation[1,3,7].

Ophthalmology and neurology were the first specialties that were interested in BTX. Since Scott's[6] work on the nonsurgical treatment of ocular strabismus with BTX-A, this toxin has been used to treat many neurological head and neck disorders in humans[1,8-12]. Since 1987, BTX-A has been used to denervate certain mimetic muscles for static facial rhytids[4,11]. Smyth first reported positive clinical results with the use of BTX-A to treat masseter muscle hypertrophy in 1994[8].

BTX-A received Food and Drug Administration (FDA) approval for treatment of strabismus and blepharospasm in 1989, cervical dystonia in 2000, and cosmetic treatment of glabellar wrinkles in 2002[4,11,13]. BTX-A application can achieve a short-term correction of muscular activities with very few side effects if applied in an accurate and controlled manner[1,2,11].

BTX is contraindicated in patients with neuromuscular junctional disorders (e.g., myasthenia gravis), or who are pregnant or lactating. The effects of BTX may be potentiated by aminoglycoside antibiotics or any other drugs that interfere with the neuromuscular transmission[3,5,9]. The standard unit for measuring the potency of the commercially available toxin is derived from a mouse assay/ mouse unit (MU). In this assay, 1 U of botulinum toxin is the amount that kills 50 percent of a group of 18 to 20 female Swiss-Webster mice[7,11,12]. The American product named Botox® (Pharm Allergan Inc., Irvine, CA, USA) is supplied in vials of 100 MU while the English product named Dysport® (Speywood Pharmaceuticals Ltd., Maidenhead, UK) is supplied in vials of 500 MU. The effect of Botox® is apparently four times higher than that of Dysport®. Thus, 100 units of Dysport® correspond to approximately 25 units of Botox®[3]. The median lethal dose (LD_{50}) of BTX-A for humans has been calculated to be 2,500 to 3,000 units[7]. The treatment dosage for facial cosmetic procedures is approximately $1/100^{th}$ of LD_{50}; therefore, there is a strict therapeutic index[4,12] .

The popularity of BTX has been increasing over the years in many disciplines of medicine, while more recently, its complications and adverse effects are widely discussed[1,11]. At this point, the need for research at the cellular level has been raised. However, there is no sufficient basic ultrastructural research about the muscular histological effects of BTX injections[14-19]. Additionally, BTX-A-induced morphological changes that underlie its therapeutic alterations on the muscle tone of the masticatory muscles, have not been described. It is controversial whether BTX causes muscle degeneration to some extent, in the course of therapy[17,18,20-23].

Recent studies have shown degeneration related to apoptotic action[17] of BTX on the masseter muscle and morphologic changes associated with a decrease in muscle fiber proteins[18]. Kim et al also examined the effects of BTX injected into the masseter muscle on the developing rat mandible and demonstrated apoptosis of the condylar cartilage[14]. In this context, the aim of the current study was to elucidate the ultrastructural effects of BTX injected into the masseter and gluteal muscles using histologic evidence.

Materials and methods

A total of 30 male 5-6-month-old Sprague-Dawley rats weighing 270 ± 30 g were used for this study. The rats were randomly divided into an experimental (n =15) and control (n =15) groups. They were housed in individual cages in a temperature-controlled environment (22 °C) with a 12:12 h light-dark cycle. All rats were fed standard pellet food (Istanbul Yem Sanayi, Turkey) and *ad libitum* tap water. Animal protocols and procedures were approved by the Istanbul University Animal Care and Use Committee (IU-DETAE - 48-004, 29/01/2004). We also followed National Institutes of Health guidelines for the care and use of laboratory animals (24.6.2004-5199). All experiments were performed at Istanbul University, Institute of Experimental Science Laboratories (DETAE, Istanbul, Turkey) from February until August 2004. All efforts were made to minimize the number of animals used and their suffering. The rats were anesthetized using an intraperitoneal injection of 60 mg/kg Ketamine Hydrochloride (Ketanest®, Parke Davis, Berlin, Germany). The masseter and gluteal muscles of the rats in the experimental group were injected with a single dose of BTX-A (Botox®, Allergan Herbert, Irvine, Calif, USA) in normal saline (0.5 U/0.1 ml), while those in the control group were injected with 0.1 ml normal saline[15]. BTX-A was injected to the superficial portion of the masseter and the middle third of gluteal muscles with a 33-gauge hypodermic needle.

At the 12^{th} week, the animals were re-anesthetized, for sampling for the light and electron microscopic studies, and 1 x 1 cm muscle specimens were biopsied from the areas that were previously injected. Also, the sciatic nerves were exposed through a gluteal muscle-splitting incision using aseptic technique, and 3-cm sections of the sciatic nerves were obtained. After the biopsy procedures, all animals were sacrificed by overdose (100 mg/kg) of intraperitoneal sodium pentobarbital anesthesia. The fresh biopsy specimens were fixed in 2.5 % (w/v) glutaraldehyde, post-fixed with osmium tetroxide, and embedded in Epon-812 for light and electron microscopic evaluation, respectively. One-micrometer-thick sections of the specimens were stained with toluidine blue and examined by light microscopy. For electron microscopy, thin sections cut by ultramicrotome (Leica, Germany) were taken on 75 mesh copper grids and stained with uranyl-acetate and lead citrate for contrast enhancement and evaluated with Jeol 1010-B electron microscope and measurements were performed via SIS digital imaging system and Analysis Software (Germany).

Quantitive parameters such as the thickness of muscle fibers were evaluated using the light microscope and qualitative assessments including sarcosomal degeneration, sarcomere deformation, glycogen content, the intensity of connective tissue around muscle and nerve fibers, and T tubule features were evaluated using light and electron microscope, respectively. We paired BTX-A (+) and BTX-A (-) samples statistically.

Statistical analyses were performed with the IBM SPSS Statistics software, version 21.0 (IBM SPSS, IBM Corp., Armonk, NY, USA). Shapiro-Wilks test of normality was done to ensure the dependent variable was approximately normally distributed within each group. Parametric independent-samples t-test was used for statistical analyses. A p value less than 0.05 was considered statistically significant. No relationship between the observations in each group or between the groups was observed. Levene's test for equality of variances indicated the variances of the two groups we were measuring were equal in the population.

Results

Increase in the collagen fibers forming perimysium around the striated muscle cells (Figure 1, left image) was observed and the diameters of many myofibers were found to be decreased compared with BTX-A (-) controls (Figure 1, right image). No abnormality was observed in the structural features of the myofibers in BTX-A (-) controls (Figure 2) while atrophic changes in the myofibrils characterized by a decrease in the myofibrillar diameters and myofibrillolysis, and dilatations in the terminal cisternae and T-tubules, and disorganized Z bands were observed in BTX-A (+) group by electron microscopy (Figure 3).

In BTX-A (+) group, vacuolar appearance was detected in many striated muscle cells (myofibers) by both light and electron microscopy (Figure 4). Electron microscopy also detected these vacuoles and revealed that they appeared as a result of dilatation in the sarcoplasmic reticulum cisternae and to some extent mitochondrial swelling.

On the other hand, in addition to atrophic changes, obliteration of mitochondrial cristae and abnormal mitochondrial along with autophagic vacuoles demonstrating myelin-like inclusions were observed (Figure 5 right and left images, respectively). Such inclusions were observed in the proximity of the muscle cell nuclei. No morphologic difference in sciatic nerve fibers was detected, and myelin sheaths of axon structures were intact in both groups (Figure 6).

Statistically, diameters of the muscle fibers in bundles and fascicles of the muscles were significantly decreased in BTX-A (+) group ($p < 0.001$) (Figure 7, Figure 8). The difference between masseter and gluteal groups was not statistically significant. Both masseter and gluteal muscles were affected to the same extent by BTX-A injections.

Discussion

In this study, we have used histological techniques including light and electron microscopy, and we found that most of the morphological changes in muscle after been injected with BTX-A, were related to atrophy. This part of our results regarding muscle atrophic changes are in agreement with similar results with previous publications, while our findings demonstrating muscle degeneration is controversial[14,17,18,20-23].

Our findings of mitochondrial swelling, decrease in muscle fiber diameters and glycogen content were extensively observed in the BTX-A (+) group. These observations were similar to those of the study by Capra et al[21] who pointed out that few changes could be found as early phase effect and myofibrillar dissolution, aberrations in Z-line, and enlarged mitochondria as mid-term results. Also, they noted a decrease in the size of muscle fibers. Our results were in accordance with the results by Capra et al[21]. Porter et al[24] showed with light and electron microscopy nonselective atrophy in almost all muscle fibers in 7-84 days. This work, just like ours, depicted structural muscle changes as reversible, with no apparent long-term consequences[24]. The most extended ultrastructural study following BTX injections was a 30-week-study carried out by Hassan et al[22] that showed muscle fiber atrophy and vacuoles of variable size in the sarcoplasm, myofibrillar structural changes and they also observed minimal pre-degenerative changes in mitochondria which were similar to our findings. On the other hand, our findings like loss of mitochondria cristae can be regarded as minimal pre-degenerative changes indicating an injury on the myofibers. But according to our findings, these changes were relatively few compared to the atrophic changes.

We observed few but prominent autophagic vacuoles in some fibers related to cellular damage. Autophagy is a somewhat different pathway for cellular injury, and this finding can be interpreted in the light of data from recent research indicating apoptosis in muscle cells exposed to BTX-A[14,17].

While most of the recent and previous studies do not support any degenerative changes of the injected muscle, there are studies demonstrating degeneration in a cellular level[17,18,20-22]. Monn et al[17] showed that the morphological changes of mitochondria were evident at 12 weeks after injection. However destruction of mitochondria structure and subsequent autophagy were observed at two weeks after BTX-A injection, muscle fiber had large mitochondria with loosely packed cristae, and regional destruction of myofibres was found in the 5 U BTX-A group. Also, they showed that this type of damage was more extensive in the 10 U BTX-A group at 12 weeks after injection[17]. Direct action of BTX-A in skeletal muscle is relatively rapid. BTX-A injection induces dramatic transcriptional adaptation occurring at one week with an activation of genes in competing pathways of repair and atrophy, gene-related impaired mitochondrial biogenesis[25]. Experimental studies demonstrated degenerative changes of the masseter muscle within two weeks following BTX-A injection. It was also shown that high doses of BTX-A could cause severe degenerative changes leading to interfiber spaces[18]. As the muscle cell is a multinucleated cell, denervation-induced apoptosis eliminates individual nuclei without destroying the entire fiber[20].

In the literature, early effects of BTX-A are investigated in the second and fourth weeks while late effects are examined in the 12th weeks [14,17,18,23], thus we sacrifised the animals on the 12th week. Clinically, the therapeutic effects of BTX-A injections are usually noted several days after the injection. In general, muscle returns to normal functioning after about four to six months. In some studies, 22-31 % muscle atrophy was observed that decreased in the first 3 months[26,27] while other researchers found that induced muscle atrophy can remain stable for 24-25 months[9]. But synaptic remodeling begins in six to 14 days, and between the fourth and twelfth weeks, nerve function recovery is completed[15,19,20,22,23]. For this reason, we extended our experiments for a maximum of 12 weeks.

In our experiments, we have used the most widely used BTX preparation. BTX-A is the most common commercially used type of BTX, but clinical experience with types B, C, and F is also increasing[1,3,4,7]. Following approval of BTX-A by FDA for use as a therapeutic agent in patients with strabismus, blepharospasm, and other facial nerve disorders, including hemifacial spasm, BTX-A has been widely used in oral and maxillofacial surgery for the treatment of temporoman-

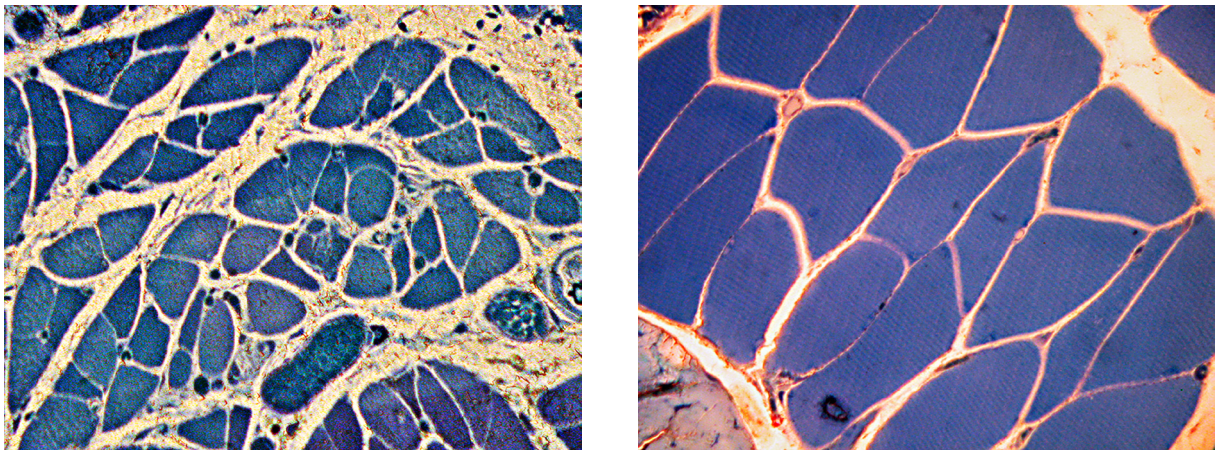

Figure 1: Light microscopy images demonstrating an increase in the connective tissue sheaths of the masseter muscle fibers in samples obtained from the animals injected with a single 0.5 U dose of botulinum toxin type A [BTX-A (+) group] (left image) and the animals injected with normal saline [BTX-A (-) group] (right image) (x200, semi-thin section, toluidine blue stain).

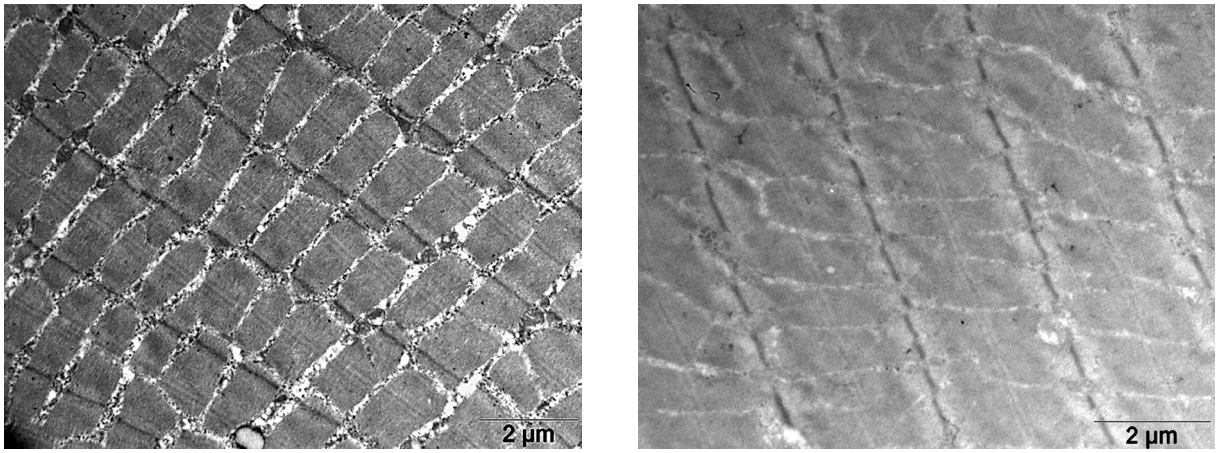

Figure 2: Electron microscopy images demonstrating normal structural features of myofibers of the gluteal (left image) and masseter (right image) muscles in samples obtained from the animals injected with normal saline [BTX-A (-)] of the control group.

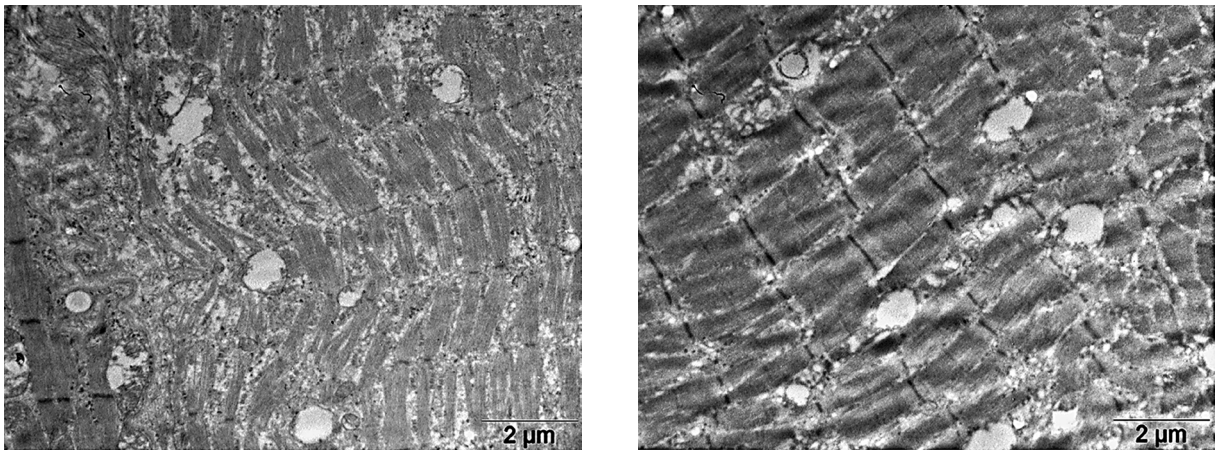

Figure 3: Electron microscopy images demonstrating myofibrillolysis and dilatations in the terminal cisternae and T-tubules and aberrations, and disorganization in Z bands in atrophied fibers of both gluteal (left image) and masseter (right image) muscles in samples obtained from the animals injected with a single 0.5 U dose of botulinum toxin type A [BTX-A (+) group].

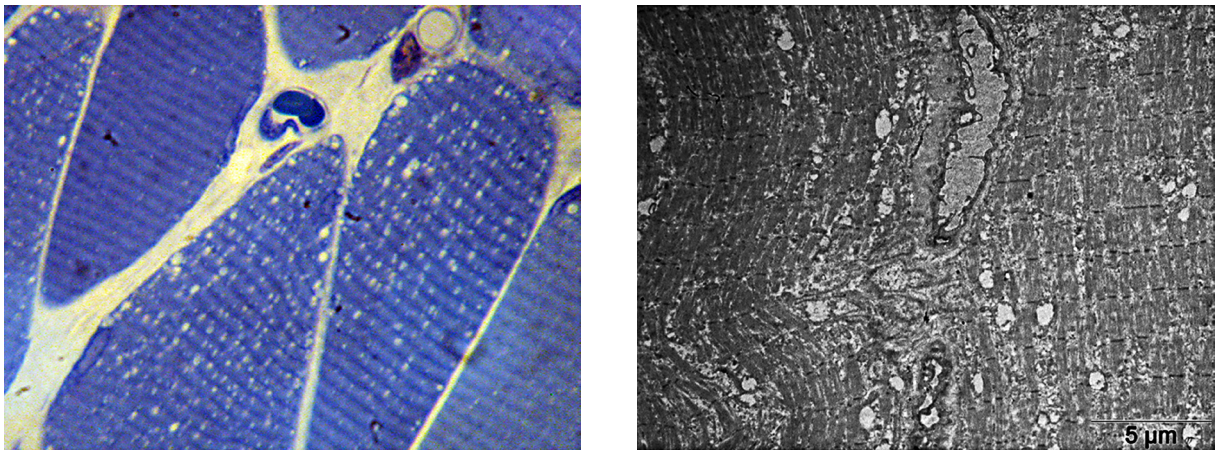

Figure 4: Light microscopy (left image, x600, left semi-thin section, toluidine blue stain) and electron microscopy (right image) appearance showing vacuolization associated with atrophy in the myofibers of gluteal muscles in samples obtained from the animals injected with a single 0.5 U dose of botulinum toxin type A [BTX-A (+) group].

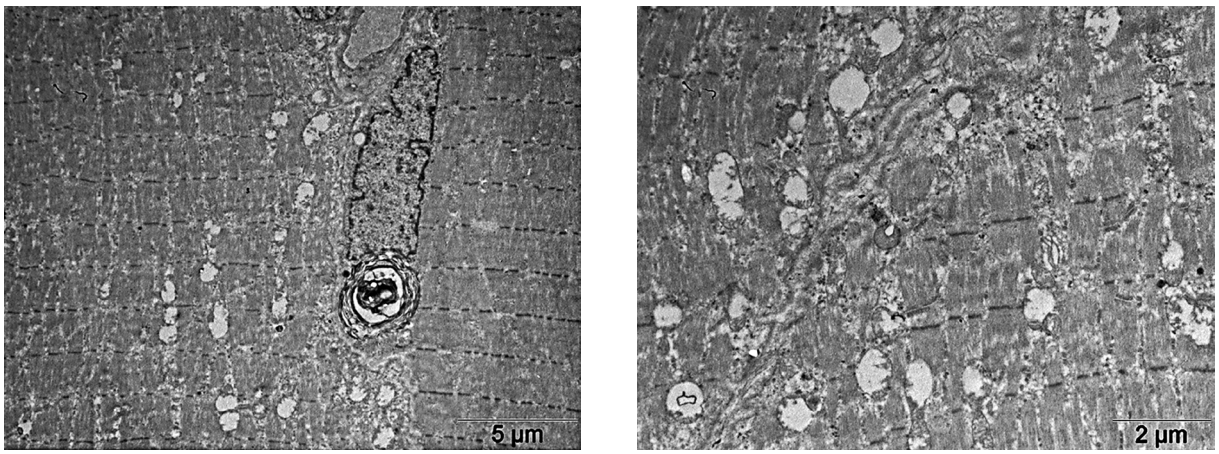

Figure 5: Electron microscopy images demonstrating autophagic vacuoles characterized by myeline-like inclusions observed in the proximity of muscle cell nuclei (left image) and dilatations in the sarcoplasmic reticulum and loss of mitochondrial cristae (right image) in gluteal muscle fibers obtained from the animals injected with a single 0.5 U dose of botulinum toxin type A [BTX-A (+) group].

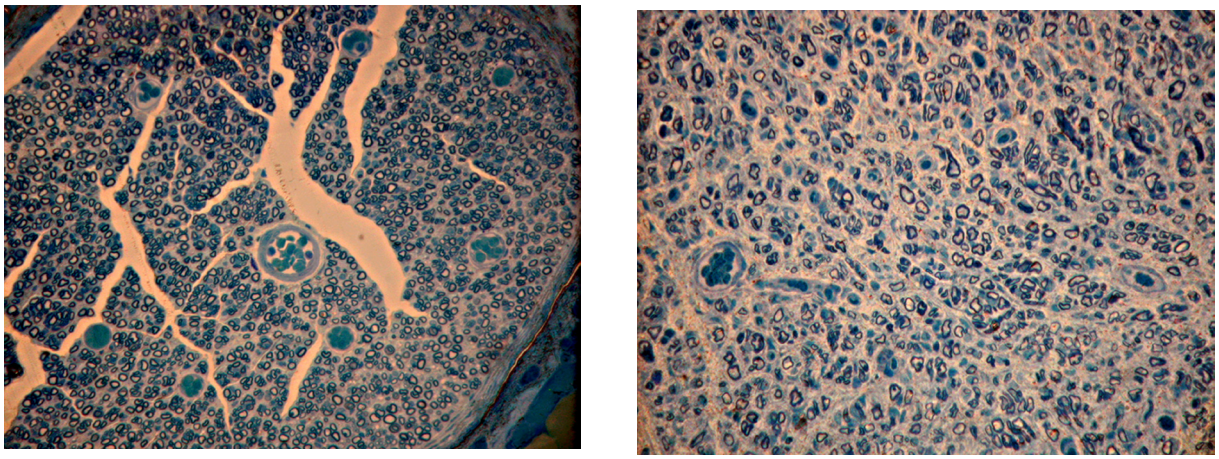

Figure 6: Light microscopy images of the sciatic nerve showing normal axonal fibers with intact myeline sheath in samples obtained from the animals injected with normal saline [BTX-A (-) group] (left image) (x200, semi-thin section) and the animals injected with a single 0.5 U dose of botulinum toxin type A [BTX-A (+) group] (right image) (x250, semi-thin section, toluidine blue stain).

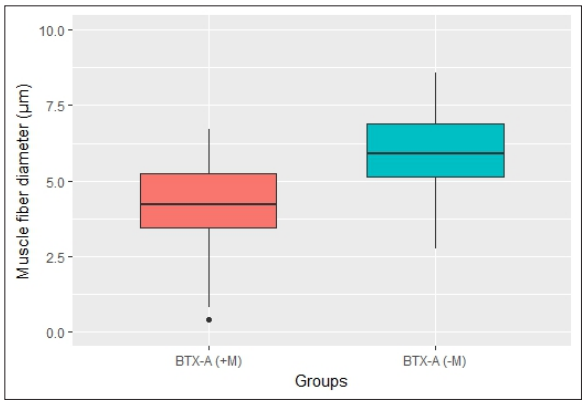

Figure 7: Effects of botulinum toxin type A on muscle fiber diameter in the masseter muscle.
BTX-A (+M): samples obtained from masseter muscle of animals injected with a single 0.5 U dose of botulinum toxin type A, BTX-A (-M): samples obtained from masseter muscle of animals injected with normal saline.

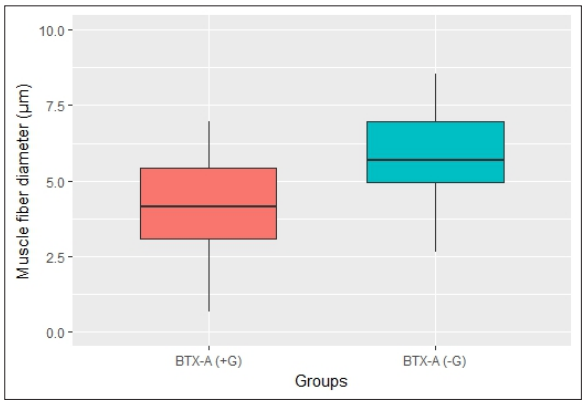

Figure 8: Effects of botulinum toxin type A on muscle fiber diameter in the guteal muscle.
BTX-A (+G): samples obtained from guteal muscle of animals injected with a single 0.5 U dose of botulinum toxin type A, BTX-A (-G): samples obtained from guteal muscle of animals injected with normal saline.

dibular disorders, masseteric hypertrophy, and treatment of masticatory system[9,13,28-30]. BTX injections are a noninvasive alternative method for treating masseteric hypertrophy[30]. Additional applications in oral and maxillofacial surgery include the denervation of hypertrophic or hyperactive masticatory muscles for cosmetic and functional purposes[28,29]. Muscle structural properties after two botulinum toxin injections demonstrate disproportionally increased effects[31]. Stem cell therapy provides new hope for patients suffering from myopathies induced by chemo-denervation atrophy by repeated local injection of botulinum toxin[32].

We followed Cicron et al[15] proposed dosage of 0.5 U which recorded no visible systemic effects. Due to the similarity of rat's sciatic nerve to that of human peripheral nerve[19], we opted to utilize rats in our study. In experimental studies which aimed to investigate the comparative effect of various doses (0.5, 2.5, 5, 10, and 25 U) of BTX-A, it was demonstrated that the high amount of BTX-A dose is a risk factor for muscular damage[2,15,17,18]. On the other hand, there are clinical studies which state that the amount of BTX-A dose is not relevant[30].

There are few studies in the literature regarding underlying ultrastructural effects of BTX treatment[17,21,22,24]; thus we decided to utilize the electron microscopy as an additional parameter to advance light microscopy findings. After BTX injections some atrophic findings were observed in the different muscles examined in the various experimental and clinical investigations. BTX produces paralysis by blocking the presynaptic release of the neurotransmitter [acetylcholine (AcH)] at the neuromuscular junction, with reversible chemical denervation of the muscle fiber, thereby inducing partial paralysis and atrophy[14,17,20-23]. To measure the atrophic changes in terms of quantitative volumetric assessment, various clinical methods have been used including computed tomography, electromyography, ultrasonography, and magnetic resonance imaging[26,27,30].

Our results demonstrating a decrease of the diameters of the muscle fibers in bundles and fascicles of muscles are in accordance with previous studies[2,18,21,22,24]. Changes of the muscle fibers following the reduction of masticatory function of the masseter by the injection of BTX-A were revealed by histological examination in our study. Other studies also revealed that the injection of BTX does not only reduce muscle weight and fiber diameter but changes the composition of muscle subtypes[2]. Moreover, BTX-A injection to the masseter muscle affects the structure of the bone and temporomandibular joint as well as causing atrophy of the muscle[14,23]. BTX applications have also shown progressive denervation like atrophy in distant muscles[33]. On the other hand, Song reported cases of patients who, seven years following BTX injections, exhibited no signs of atrophy or degeneration in muscle biopsies[34].

In the current study, nerve fiber structures displayed no clear morphological difference between the BTX-A (+) and BTX-A (-) groups. In the experimental study by Lu et al[19], even a direct intraneural injection of BTX caused no damage, and these findings are similar to ours. On the other hand, several studies have supported the minimal reversible effects of BTX-A on nerve injury[20,35,36]. Chemical denervation after BTX injections is reversible. BTX has temporary effects and the muscle is progressively reinnervated by nerve sprouting. Recovery is believed to be related to the abundant axonal sprouts that form after neuromuscular junctions are poisoned[19,22,23].

Nowadays, although BTX is used widely in medicine[37], there are discussions and concerns regarding complications and adverse effects. Phadake et al[38] has reported hospitalization around 18 % of which 53 % was serious, and fatality of 8% among 282 patients who visited the hospital in 4-year-period[38]. Another study reported that the risk of complication related to BTX injection was 33 times higher in its therapeutic application than for cosmetic cases[11]. In order to avoid and minimize BTX complications, ultrastructural effects should be taken into consideration while treating patients more professionally.

Conclusion

In this study, we have found that most of the ultrastruc-

tural morphological muscle changes following BTX-A injection, were associated with muscle atrophy. We conclude that muscular changes, predominantly related to atrophy rather than degeneration are induced by BTX-A. This finding suggests that when the effects of chemical denervation cease, the myofibers will function normally again.

Autophagic vacuoles, myofibrillolysis, and loss of mitochondrial cristae are often related to degeneration rather than atrophy at least to some extent. Our degeneration related findings suggest that further studies are needed focusing on detecting BTX-A effects on a cellular level.

Conflict of Interest

Authors have no conflict of interest to declare.

Acknowledgements

Authors would like to thank Dr. Irem Kirli Topcu and Dr. Melike Subay for their technical support.

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