401 N. Lindbergh Blvd.
St. Louis, MO 63141
Tel.: 314.993.1700, #546
Toll Free: 800.424.2841, #546
Fax: 800.708.1364
Cell: 314.283.1983
Send via email to: jbode@aaortho.org and cyoung@aaortho.org

AAO Foundation Final Report Form (a/o 6/30/2018)

In an attempt to make things a little easier for the reviewer who will read this report, please consider these two questions before this is sent for review:

- *Is this an example of your very best work, in that it provides sufficient explanation and justification, and is something otherwise worthy of publication? (We do publish the Final Report on our website, so this does need to be complete and polished.)*
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Please prepare a report that addresses the following:

Type of Award, e.g., Orthodontic Faculty Development Fellowship Award, Postdoctoral Fellowship Award, Biomedical Research Award, Center Award, Educational Innovation Award, Program Award, Research Aid Award

Orthodontic Faculty Development Fellowship Award

Name(s) of Principal Investigator(s): **Eliane H Dutra**

Institution: **University of Connecticut Health**

Title of Project: **Long-term effects of Botox injection into the muscles of mastication in the mandibular condylar cartilage**.

Period of AAOF Support (e.g. 07-01-19 to 06-30-20): **07-01-17 to 12/31/18**

Amount of Funding: **$20,000**

Summary/Abstract :

Objectives: To evaluate whether the effects of botulinum neurotoxin (Botox) injection into the masseter in the mandibular condylar cartilage (MCC) and subchondral bone are transient. Materials and Methods: Botox (0.3 U) was injected into the right masseter of 6-week-old female mice (C57BL/6; n = 16). Additionally, 16 mice were used as control and received no injections. Experimental and matching control mice were sacrificed 4 or 8 weeks after the single botox injection. Mandibles and mandibular condyles were analyzed by micro-CT and histology. Sagittal sections of condyles were stained for TRAP, toluidine blue, EdU and TUNEL.
Results: Bone volume fraction was significantly decreased on the subchondral bone of botox injected side when compared to control side and control mice, 4 and 8 weeks after injection. Furthermore, histological analysis revealed decrease in mineralization, cartilage thickness, TRAP activity and EdU positive cells in the MCC of the botox injected side, 4 and 8 weeks after injection. Conclusion: The effects of Botox injection into the masseter muscle in the MCC and subchondral bone persisted for 8 weeks after injection and were not considered transient.

Detailed results and inferences: **Accepted manuscript for publication is attached.**

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2. Describe in detail the results of your study. The intent is to share the knowledge you have generated with the AAOF and orthodontic community specifically and other who may benefit from your study. Table, Figures, Statistical Analysis and interpretation of results should be included.

Respond to the following questions:

1. Were the original, specific aims of the proposal realized? **Yes**
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    b. Was AAOF support acknowledged? **Yes**
    c. If not, are there plans to publish? If not, why not? **NA**
3. Have the results of this proposal been presented? **Yes**
    a. If so, list titles, author or co-authors of these presentation/s, year and locations: **Presented at the 2018 UConn Symposium in the University of Connecticut, Farmington, CT in May 1st 2018. Results were also presented at the 2017 Burstone Presymposium in the Indiana University in October 5th 2017.**
    b. Was AAOF support acknowledged? **Yes**
    c. If not, are there plans to do so? If not, why not? **NA**

4. To what extent have you used, or how do you intend to use, AAOF funding to further your career?

The AAOF funding has helped me to generate data for future NIDCR and AAOF grants submission and to publish key manuscripts. In addition, the AAOF support has provided me the funds to become board certified (I became certified by the American Board of Orthodontists in February of 2018).

Accounting for Project; i.e., any leftover funds, etc.

There was about $2,000 leftover

The effects of Botox injection into the masseter in the mandibular condyle are not transient

Eliane H. Dutra, DDS, MSD, PhD
Assistant Professor
Division of Orthodontics
University of Connecticut Health Center

Sumit Yadav, BDS, MDS, PhD*
Associate Professor
Division of Orthodontics
University of Connecticut Health Center

* Corresponding Author: 263 Farmington Avenue, L7063 MC1725, University of Connecticut Health Center, Farmington, CT 06030. Email: yadav_sumit17@yahoo.com

Abstract

Objectives: To evaluate whether the effects of botulinum neurotoxin (Botox) injection into the masseter in the mandibular condylar cartilage (MCC) and subchondral bone are transient. **Materials and Methods:** Botox (0.3 U) was injected into the right masseter of 6-week-old female mice (C57BL/6; n = 16). Additionally, 16 mice were used as control and received no injections. Experimental and matching control mice were sacrificed 4 or 8 weeks after the single botox injection. Mandibles and mandibular condyles were analyzed by micro-CT and histology. Sagittal sections of condyles were stained for TRAP, toluidine blue, EdU and TUNEL.

Results: Bone volume fraction was significantly decreased on the subchondral bone of botox injected side when compared to control side and control mice, 4 and 8 weeks after injection. Furthermore, histological analysis revealed decrease in mineralization, cartilage thickness, TRAP activity and EdU positive cells in the MCC of the botox injected side, 4 and 8 weeks after injection. **Conclusion:** The effects of Botox injection into the masseter muscle in the MCC and subchondral bone persisted for 8 weeks after injection and were not considered transient.

Keywords: Botulinum Toxin, Temporomandibular joint, Subchondral bone, Mineralization, Mandibular condyle

Introduction

The expanded classification of temporomandibular joint disorders (TMD) includes temporomandibular joint (TMJ) conditions, masticatory muscles disorders, headaches and abnormalities of related structures, such as coronoid hyperplasia [1]. The affected individuals suffer from functional and psychological impairments[2], placing this group of disorders as a leading cause for disability. Botulinum neurotoxin (Botox) injections into the muscles of mastication have been reported as a promising adjunct treatment for the relief of the orofacial pain correlated with TMD [2-4]. Botox has a therapeutic effect due to its actions at the neuro-muscular junction, exerting a local paralytic effect by inhibiting acetylcholine release [5]. However, blocking the contraction of the muscles of mastication by Botox injections has been shown to result in negative side effects in the craniofacial structures [6-9]. A particular concern has been raised in the effects of this treatment modality in the mandibular condyle; animal and clinical studies have reported decrease in condylar bone volume and cartilage thickness [9-11], effects that could lead to condyle fracture and TMJ degeneration.

The TMJ is a dynamic structure containing the unique fibrocartilage that responds to changes in loading demands [12-14]. The mandibular condylar cartilage (MCC) has distinct cellular zones, in which cell proliferation, chondrocyte differentiation and mineralization occur in a sequential manner [14]. The MCC also contains a non-mineralized region for resistance to compressive forces [15]. In our previous report, we found that Botox injection into the masseter of 5-week-old mice disrupted the cellularity, the matrix composition and mineral deposition within the MCC 4 weeks after a single injection [11].

The effects of Botox in muscle are typically sustained for about 4-6 months in humans and after this period additional injections are usually necessary [16]. However, there are limited studies on the long-term effects of Botox injection in the masseter and its effects on the cartilage of the TMJ. Additionally, it remains to be determined if the MCC would adjust to the altered loading pattern induced by Botox injection into the masseter. The aim of this study was: 1) to evaluate the

long-term effects of the botox on the MCC of the TMJ and; 2) to compare and contrast the effects of short-term and long-term botox after unilateral injection of Botox into the masseter muscle. In the study reported here, we hypothesized that the short-term and long-term effects on botox on the mandibular condyle will be different and short-term effects on the mandibular condyle are transient and will be diminished over time.

Materials and Methods

Mice

The Institutional Animal Care Committee of the University of Connecticut Health Center (UCHC) reviewed and approved all procedures. The experiments followed the ARRIVE guidelines [17]. We used 6-week-old females (C57BL/6; n = 32) obtained from The Jackson Laboratories (Bar Harbor, ME, USA). Mice at six weeks of age are considered in late puberty stage, while at 10 and 14 weeks (corresponding to the time points evaluated) are in adulthood (early 20 years of age if compared to human)[18]. This age was selected because 6-week-old mice are not in accelerated development at this stage, corresponding to the age of patients receiving Botox injections into the masseter.

Experimental Procedure

Botox (Botox®; Onabotulinum toxin A; Allergan, Plc; Parsippany-Troy Hills, NJ, USA) was injected into the right side masseter of experimental mice (0.3 unit, volume of 30 µl). The left side masseter of experimental mice did not receive any injection and the left condyle was considered the “control side”. Mice were anesthetized with ketamine (90 mg/kg) and Xylazine (13 mg/kg) before the injections.

We used two different endpoint to evaluate the short-term and long-term effects of Botox injection into the masseter muscle.

1) Short-term botox - 4 weeks (n = 8): Mice were euthanized 4 weeks after unilateral Botox injection into the masseter;
2) Long-term botox - 8 weeks (n =8): Animals were euthanized 8 weeks after unilateral Botox injection into the masseter;

In addition, we had a matching control group (pure control) for each of the time points (8 mice each control group). The pure control group of mice did not receive any botox injection into the masseter and represented mice that did not receive any treatment.

All mice received intraperitoneal injections of the fluorochrome labels calcein (10 µg/kg body weight) and alizarin complexone (10 µg/kg body weight), 72 and 24 hours before euthanasia. Moreover, mice were injected with the cell proliferation marker EdU (5-ethnyl-2'-deoxyuridine, Life Technologies, Grand Island, NY, USA), in a concentration of 30mg/kg body weight, 48 and 24 hours before euthanasia. Mice were euthanized at each time point by CO_2 asphyxiation and death was confirmed by cervical dislocation.

Tissue Preparation and Histology

Before dissecting the mandibles, facial tissues around the masseter and mandible were removed, exposing the cleaned masseters. Photographs of the dissected mice were performed using a digital camera (Canon EOS Rebel T3i, Canon USA, Inc.).

Mandibles were then dissected free by cutting the muscular attachment without scrapping the cartilage of the condyle and fixed in 10% Formalin for 48 hours. Fixed undecalcified mandibles were placed in 30% sucrose overnight and embedded in frozen specimen embedding medium (Shandon Cryomatrix, Thermo Scientific, Pittsburgh, PA, USA). Frozen sagittal sections of the condyles (5 µm) were performed using the Kawamoto method [19].

Micro-CT

We used micro-computerized tomography (SCANCO Medical AG, Brüttisellen,

Switzerland) to analyze the bone volume fraction (BVF (%)) of the mineralized cartilage and subchondral bone of condyles of experimental and control mice. For BVF, the whole condyle head represented 100%, and that included unmineralized and mineralized tissue. The samples were scanned in 70% ethanol, one at a time, with high resolution in a 16mm holder. Serial tomographic projections were acquired at 55kV and 145µA, with a voxel size of 6µm and 1000 projections per rotation collected at 300000µs. The DICOM images were transferred, segmented and reconstructed using the mimics software (Materialise, Belgium). In order to distinguish calcified tissue from non-calcified tissue, an automated algorithm using local threshold segmented the reconstructed grey scale images.

Histological Imaging and Staining

Slides were initially scanned by a fluorescent microscope (Axio Observer, Carl Zeiss, Thornwood, NY, USA). Next, the coverslip was removed by soaking the slides in PBS and the same sections were stained for Tartrate Resistant Acid Phosphatase (TRAP) using ELF97 (Life Technologies, Grand Island, NY, USA), generating a yellow fluorescent signal. After imaging for TRAP, sections were stained for EdU (ClickiT® EdU Alexa Fluor 555 HCS kit, Life Technologies, Grand Island, NY, USA) and imaged. Finally the same slide was stained for toluidine blue (TB), and re-imaged. Different slides were also stained for TUNEL (DeadEnd™ Fluorometric TUNEL System, Promega, Madison, WI, USA).

Histological analysis and Quantification

Saggital sections of condyles of 8 mice per group were quantified. Three sections per mouse were included in the quantification.

Quantification of fluorescent staining and fluorochrome labels was performed by Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA, USA). We examined TRAP (Fig. 2), calcein (Fig. 3) and alizarin complexone (Fig. 3) in the subchondral bone of condyles by counting

the number of yellow pixels (TRAP staining, generated by ELF97), green pixels (calcein) and red pixels (alizarin complexone) in each separate image and dividing it by the total number of pixels in the subchondral bone region (calculating the percentage of positive pixels over the region). Similarly, cell proliferation (Fig. 5) was quantified by counting EdU (yellow) and DAPI (blue) positive pixels in the mandibular condylar cartilage (MCC) and then calculating the percentage of EdU positive pixels over DAPI positive pixels. Moreover, cell apoptosis (Fig. 5) was quantified by calculating the percentage of TUNEL (green) positive pixels over DAPI (blue) positive pixels in the MCC.

Cartilage thickness (Fig. 4) was analyzed using Digimizer® Image software (MedCalc Software, Belgium); measurements were performed from the outer cellular layer of MCC to the tidemark (in five different locations in the entire MCC) and an average for each image was calculated.

Statistical analysis

Descriptive statistics were used to examine the distribution of bone volume fraction and histological analysis. Outcomes were compared between the Botox injected side, control side and matching pure control groups. Statistically significant differences among means were determined by One-way ANOVA. A p-value of < 0.05 was deemed to be statistically significant (Table 1). Statistical analyses were computed using GraphPad Prism (GraphPad Software, Inc, La Jolla, CA, USA).

Results

Reduction in masseter volume after unilateral injection of Botox into the masseter of mice is more noticeable 8 weeks after injection

Botox injections into the masseter are also used as cosmetic procedure to reduce the thickness of the masseter [20]. In order to validate our method in terms of reduction of the masseter

muscle, we took photographs of the dissected experimental mice to observe changes in masseter volume, 4 or 8 weeks after injection. We noticed a mild reduction in masseter volume at the Botox injected side (right side) in comparison to contralateral side 4 weeks after injection (Fig. 1A), but a more prominent reduction in muscle size 8 weeks after unilateral injection (Fig. 1B).

The reduced mineralization in the mandibular condyle induced by Botox injection into the masseter is not transient

We used Micro-CT analysis to compare the bone volume of the mineralized cartilage and the subchondral bone between botox injected side, control side and control groups at each time point. Unilateral injection of Botox into the masseter led to a decrease in mineralization and bone remodeling in the mandibular condyle of experimental mice, 4 and 8 weeks after injection. We observed a 10% decrease in the BVF in the Botox injected side in comparison to control side and control mice 4 weeks after injection (Fig. 2A-D and Table 1). The observed reduction in BFV persisted for 8 weeks after injection (Fig. 2E-H and Table 1). To further understand the changes in mineralization, we analyzed TRAP activity and the mineralization labels calcein and alizarin complexone within the subchondral bone. There was a significant decrease in TRAP activity in the Botox injected side in comparison to control side and pure control, as revealed by approximately 60% decrease in TRAP positive pixels in the Botox side in comparison to controls 4 weeks after injection (Fig. 2I-M and Table 1). Likewise, there was approximately 50% reduction in TRAP activity 8 weeks after unilateral injection (Fig. 2N-Q and Table 1). Next, we analyzed the fluorochrome labels calcein and alizarin complexone. Our quantification revealed a substantial reduction of 80% in calcein labeling (Fig. 3A-C and 3G and Table 1) and 90% decrease in alizarin complexone (Fig. 3A-C and 3H and Table 1) in the subchondral region, 4 weeks after Botox injection. Analysis of mineralization labeling 8 weeks after unilateral Botox injection into the masseter revealed that the reduced mineral deposition was not reversed with time. We noticed a 50% reduction in calcein at the Botox injected side in comparison to control side and a 70%

reduction when the botox injected side was compared to pure control, 8 weeks after injection (Fig. 3D-F and 3I and Table 1). Similarly, Alizarin complexone labeling was about 65% decreased 8 weeks after injection (Fig. 3D-F and 3J and Table 1).

The reduced mandibular condyle cartilage thickness after Botox injection into the masseter is not transitory

We next evaluated the structural and cellular changes within the MCC, 4 or 8 weeks after unilateral Botox injection into the masseter. We started by analyzing the cartilage thickness by Toluidine blue (TB) staining. Quantification of the TB distance mapping showed a significant reduction in cartilage thickness at the Botox injected side in comparison to control side and pure control group 4 weeks after injection (Fig. 4A-D and Table 1). Interestingly, cartilage thickness analysis 8 weeks after injection suggested that not only the Botox injected side, but also the contralateral (control side), were affected (Fig. 4E-H and Table 1).

The decrease in chondrocyte proliferation due to Botox injection into the masseter persists until 8 weeks after injection, but cell apoptosis is partially reversed

In addition, we studied cell proliferation and apoptosis in the MCC after Botox injection into the masseter. We found a significant reduction in the number of EdU positive cells at the proliferative zone of the MCC (outer layer), 4 and 8 weeks after injection (Fig. 5A-H and Table 1). Subsequently, cell apoptosis was evaluated by TUNEL staining. In all groups, TUNEL positive cells were observed mostly at the prehypertrophic layer of the MCC (Fig. 5I-L and Fig. 5N-P). Surprisingly, there was no statistically significant difference between Botox side and control side, but there was significantly less TUNEL positive cells at the MCC of these groups in comparison to pure control 4 weeks after injection (Fig. 5A-D and Table 1). TUNEL quantification 8 weeks after injection revealed a similar trend, but there was no statistically significant difference between

any of the groups, suggesting that the effects of Botox injection into the masseter in cell apoptosis were reversed 8 weeks after injection (Fig. 5N-Q and Table 1).

Discussion

Contrary to our hypothesis, the effects of botox are not transient and may persist over longer periods of time. Botox has been used as a palliative treatment to improve orofacial pain in patients with TMD. We have found that unilateral injection of Botox into the masseter of 5-week-old mice caused dramatic changes in the MCC, 4 weeks after injection [11]. In the present study, we aimed to evaluate the long-term effects of this procedure in the MCC and subchondral bone of 6-week-old mice. We have found that most of the effects observed 4 weeks subsequently to Botox injection persisted to 8 weeks.

We observed muscle atrophy in response to unilateral botox injection into the masseter muscle, which was more severe at 8 weeks when compared to 4 weeks indicating that the acute muscle paralysis was not transient. It has been shown that the electrical activity of the muscle is diminished within several hours of injection of botox and the muscular activity is completely inhibited by 18 hours [21]. The recovery period for the neuromuscular paralysis induced by Botulinum Neurotoxin type A in rodents is shorter than in humans, with reestablishment of muscular contraction as early as 1 month after injection [22,23]. Furthermore, the duration of muscular paralysis seems to be dependent on the dosage of the botox injection [24]. Our dosage of 0.3 units into the masseter muscle were within the range of low dose for rodents [25] .

The long-term effect of Botox injections into the masseter of the rabbits has been studied by Rafferty *et al.* [9], the authors have found decreased bone volume of the subchondral bone at the injected side, 4 and 12 weeks after injection, suggesting that the bone loss caused by masseter paralysis persists with time. Our results are consistent with this finding, we observed

reduced bone volume and a severe decrease in bone turnover, 4 and 8 weeks after unilateral injection into the masseter of mice.

The decrease in bone remodeling was illustrated by a reduction in osteoclast activity and osteoblastic activity (mineral deposition), represented by a significant reduction in the number of osteoclasts and calcein and alizarin complexone labeling. These results suggest that the decrease in bone volume at the mandibular condyle seems to be a result of a decrease in osteoblast activity (bone labeling) rather than an increase in bone resorption, at 4 and 8 weeks after botox injection. However, it is also possible that the reduction in bone volume of the mandibular condyle is due to increase in osteoclastogenesis, which may have occurred within few days after botox injection. Aliprantis *et al*.[26] studied osteoclastogenesis in the limb of mice that received a single Botox injection into the calf muscle. Interestingly, they noticed a remarkable increase in the number of osteoclasts at the injected side, in comparison to contralateral side, 5 days after injection, suggesting increased local bone resorption secondary to Botox injection. Furthermore, Ausk *et al.* suggested that Botox-induced tibia paralysis in mice causes infiltration of inflammatory cells within the adjacent marrow 24 hours following injection, leading to an increase in osteoclast fusion and expression of pro-osteoclastic genes 72 hours after paralysis [27]. Additionally, it has been shown that the bone loss in murine models due to muscle atrophy is both due to decreased osteoblastic activity and increased osteoclastic activity [28]. The masseter muscle provides the means by which the mandible is elevated and protruded thus loading the mandibular condyle and therefore directly modulating the mechanical environment of the cartilage and the subchondral bone of the TMJ. Our results showed that the mineralized tissue of the mandibular condyle is highly sensitive to alteration in the mechanical environment and responds immediately and non-transiently to acute muscular paralysis.

We observed a reduction in cartilage thickness, initially noticed at the injected side only at 4 weeks but at both injected and contralateral sides at 8 weeks, suggesting that both sides are affected and altered loaded as a result of unilateral Botox injection into the masseter. The long-

term effects of Botox injection into the masseter in the mandibular cartilage has been studied in rabbits, however no effects on the cartilage thickness were noticed as a result of treatment [29]. The short-term results in the present study are consistent with our previous report in 5-week-old mice [11], in which we observed not only a reduction in cartilage thickness, but also a diminished cartilage width as a consequence of masseter paralysis.

The MCC is formed by four distinct zones. The outer layer, or superficial zone, dissipates loading strains. The second layer is the proliferative zone, where most of cell proliferation in response to different loading demands occurs. The third zone is comprised by mature chondrocytes, cells that still have the potential to proliferate. The deepest and fourth layer is formed by hypertrophic chondrocytes, which die, have their cytoplasm evacuated and undergo mineralization [14,30-32]. Our EdU proliferation assay showed significantly decreased cell proliferation in the MCC of injected side, consistent with the effects seen after TMJ unloading experiments in mice [13,14], 4 and 8 weeks after injection. The non-transient reduction in cell proliferation suggests that the unloading effect in the MCC likely persists for the period wherein Botox is exerting its effect in the muscle. Chondrocyte apoptosis in the MCC is associated with the transition from chondrogenesis to osteogenesis [14]. Consistent with a reduction in mineralization, we found a significant decrease in TUNEL positive cells at the Botox injected side, 4 weeks after injection. However, we did not find a statistically significant difference in apoptotic activity 8 weeks after injection.

There are limited clinical studies on the long-term effects of Botox treatment for TMDs in the mandibular condyle. Raphael *et al*., in a retrospective study, evaluated by cone-beam computed tomography, condyles of women who received at least one injection of Botox into the muscles of mastication for the treatment of the symptoms correlated with orofacial pain. Decreased bone density was observed in all women who received Botox injections [10]. Recently, a case report showed unilateral condylar degeneration in a 55-year-old female patient with history

of TMD and Meige Syndrome. Condylar erosion was noticed after the patient received quarterly injections of Botox into the masseter for a period of one year [33].

One of the limitations of this study was the use of 8 weeks as our long-term time point, given that the paralysis effect could last up to more than 2 months in rodents [23]. Our future studies will focus on the effects of multiple injections and the combination of injection in additional muscles.

Conclusions

The long-term effects of Botox injection into the masseter in the mandibular condyle of mice presented here suggests that the MCC does not adapt to the changing in loading demand caused by masseter paralysis:

1) There is a significant reduction in the bone volume and subchondral remodeling of the mandibular condyle at 4 weeks and 8 weeks after botox injection.
2) There is a significant decrease in cartilage thickness and cellular proliferation at the MCC at 4 weeks and 8 weeks after botox injection into the masseter muscle.

Competing Interests: The authors have declared that no competing interests exists.

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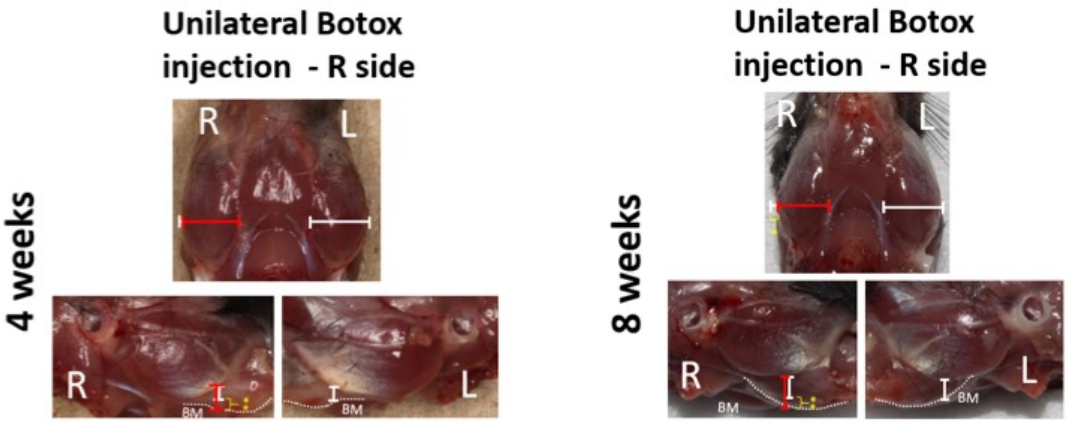

Figure 1: Changes in volume of masseter after Botox injection, 4 and 8 weeks after injection. A) Photograph of a dissected experimental mice 4 weeks after unilateral injection at the right (R) side observed from the inferior view. The white bar represent the lateral-medial length of the contraletal side (L), while the red bar represent the length at the injected side (R). The difference between the two sides are represented by overlapping the two bars at the R side. Right side masseter is slightly smaller in injected side (R) in comparision to contraleral side (L) 4 weeks after injection. **B,C)** Lateral view of dissected muscles. The distance between the inferior portion of the masseter and the border of mandible is showed. The white bar represents the contralateral side **(B)** and, the red bar, the injected side **(C)**. There is a much larger distance in the injected side (R) in comparison to contralateral (L), suggesting that the masseter was shorter in the Botox injected side. **D)** Photograph of a dissected experimental mice 8 weeks after unilateral injection at the right (R) side observed from the inferior view. The difference between the two sides are represented by overlapping the two bars at the R side. Right side masseter is substantially smaller in injected side (R) in comparision to contraleteral side (L) 8 weeks after injection. **E,F)** Lateral view of dissected muscles. The distance between the inferior portion of the masseter and the border of mandible is showed. The white bar represents the contralateral side **(E)** and, the red bar, the injected side **(F)**. Similarly to what was observed after 4 weels, the distance in the injected

side (R) was much larger than the contralateral side (L), suggesting that the masseter was still shorter in the Botox injected side after 8 weeks.

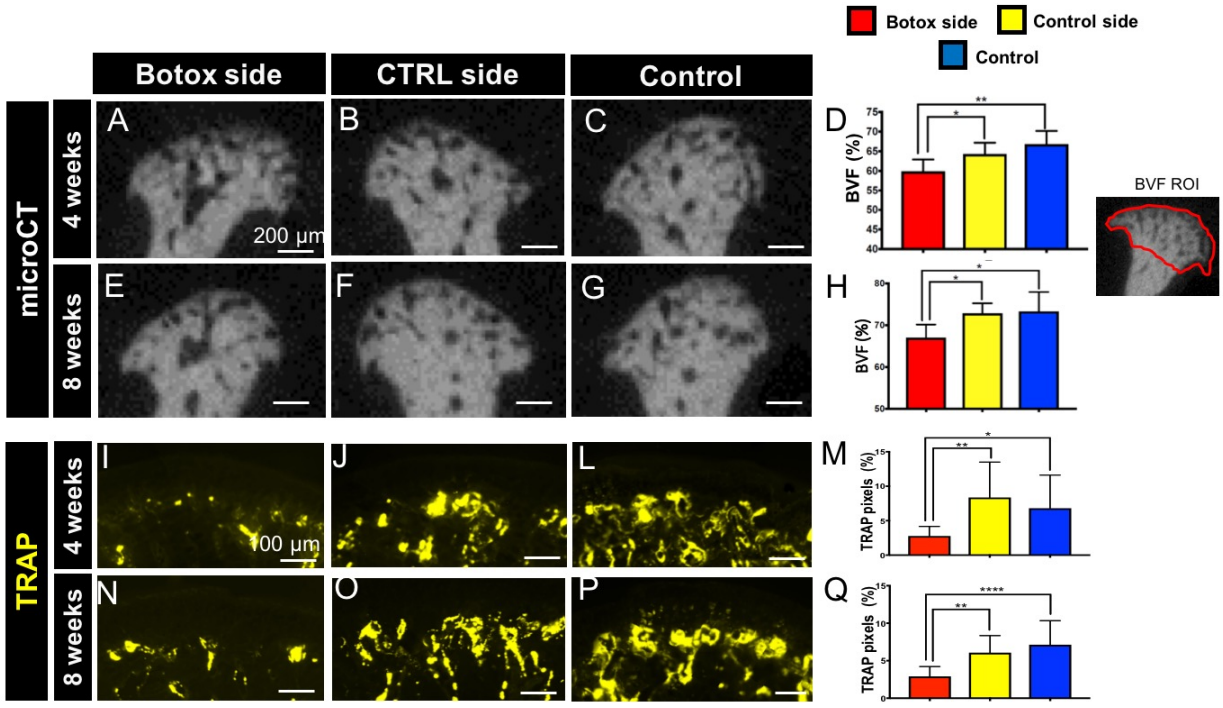

Figure 2: Reduced bone volume and bone remodeling of the condyle at the Botox injected side, 4 and 8 weeks after injection into the masseter. Coronal micro-CT images of condyles of Botox injected side **(A, E)**, control side **(B, F)** and pure control mice **(C, G),** 4 weeks **(A, B, C)** and 8 weeks **(E, F, G)** after unilateral injection. **D)** Quantification of bone volume fraction (BVF) 4 weeks after injection. **H)** Quantification of BVF 8 weeks after injection. Sagittal sections of condyles stained for TRAP: Botox injected side **(I, N)**, control side **(J, O)** and pure control mice **(L, P),** 4 weeks **(I, J, CL)** and 8 weeks **(N, O, P)** after unilateral injection. **M)** Quantification of TRAP positive pixels (yellow) over subchondral bone area, 4 weeks after injection. **Q)** Quantification of TRAP positive pixels (yellow) 8 weeks after injection. Histograms **(D, H, M, Q)** represent means ± SD for n = 8 per group. Significant difference between groups (*p < 0.05,

p<0.05, ****p<0.0001). Scale bar: **(A-G) 200µm, **(I-P)** 100µm. BVF ROI: Bone volume fraction region of interest.

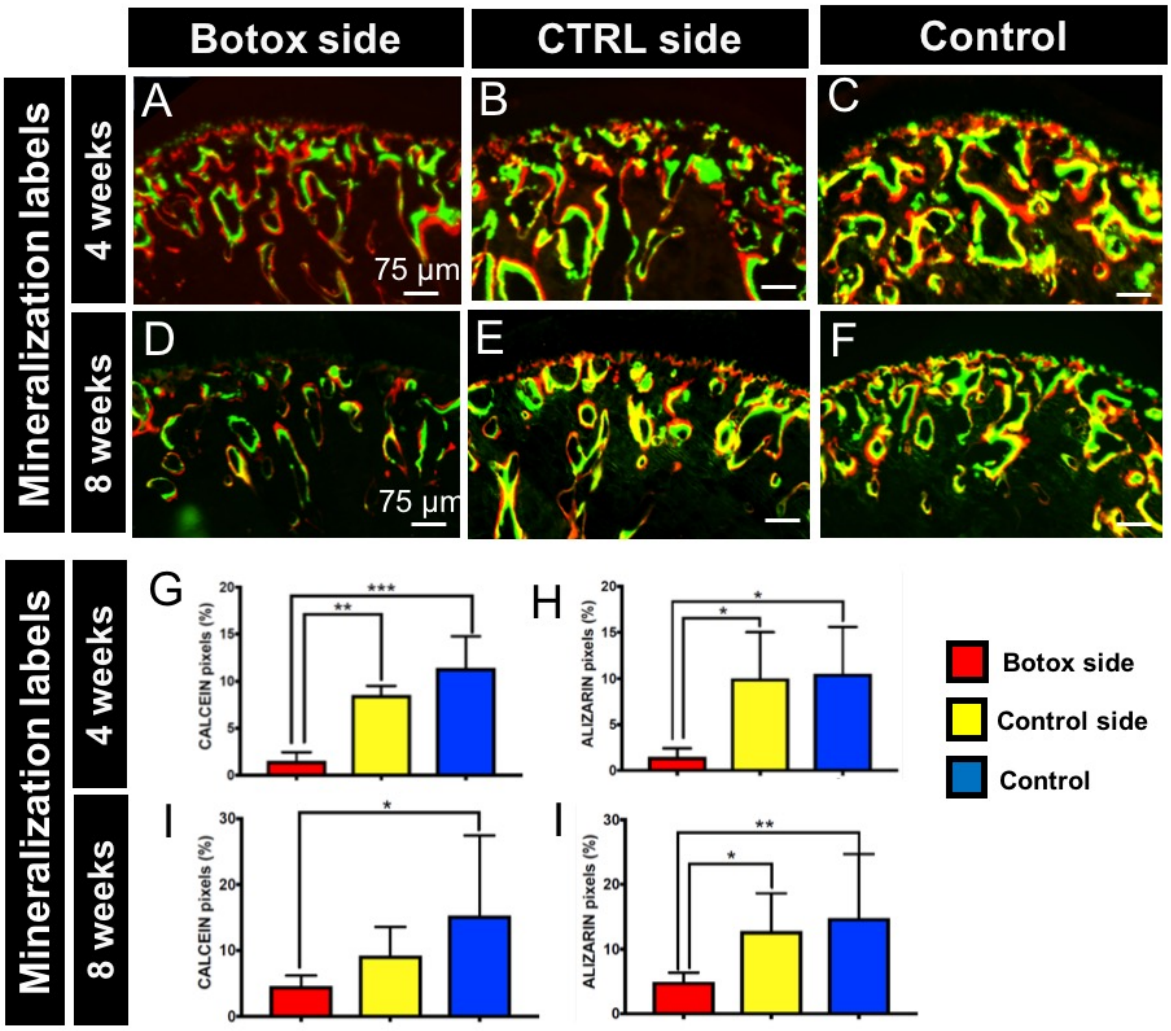

Figure 3: Decreased mineralization in the subchondral region at the Botox injected side, 4 and 8 weeks after injection into the masseter. Sagittal sections of condyles of Botox injected side **(A, D)**, control side **(B, E)** and pure control mice **(C, F),** 4 weeks **(A, B, C)** and 8 weeks **(D, E, F)** after unilateral injection into the masseter. Quantification of fluorochrome labels positive pixels (green - calcein; red – alizarin complexone) 4 and 8 weeks after injection: **G)** calcein after 4 weeks, **H)** Alizarin after 4 weeks, **I)** calcein after 8 weeks, **J)** alizarin complexone after 8 weeks.

Histograms **(G-J)** represent means ± SD for n = 8 per group. Significant difference between groups (*p < 0.05, **p<0.05, ***p<0.005). Scale bar = 75µm.

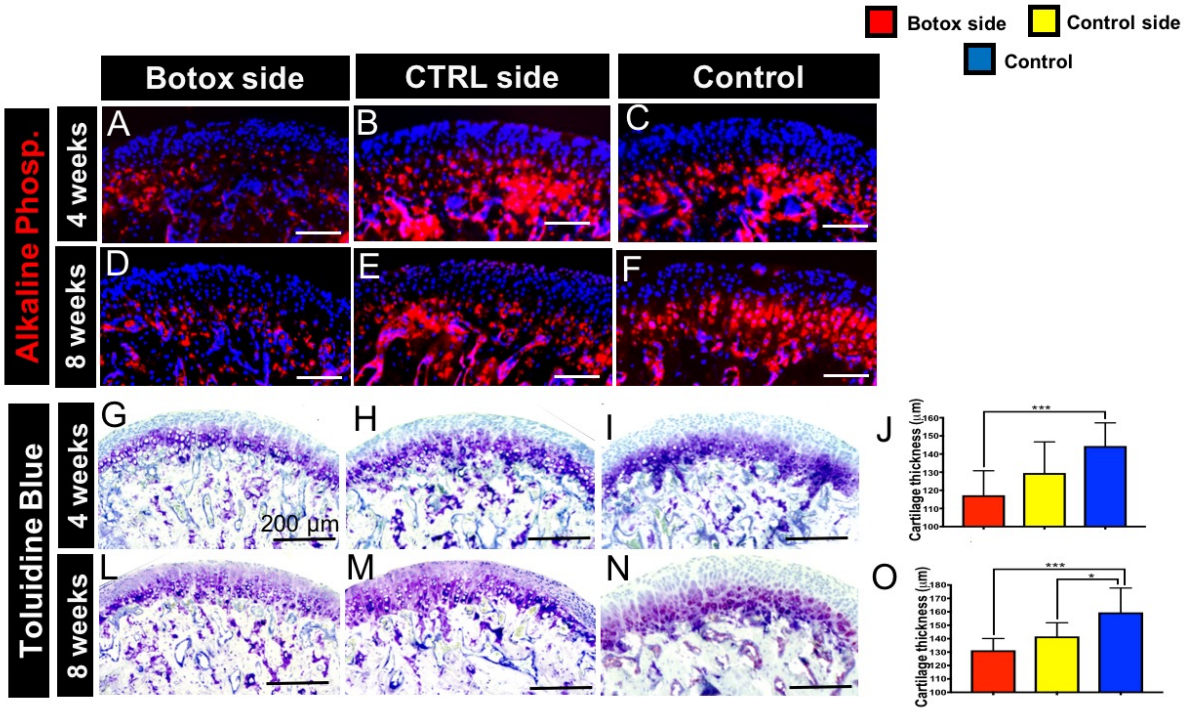

Figure 4: Reduced cartilage thickness of the mandibular condyle at the Botox injected side, 4 and 8 weeks after injection into the masseter. Sagittal sections of condyles stained for toluidine blue: Botox injected side **(A, E)**, control side **(B, F)** and pure control mice **(C, G),** 4 weeks **(A, B, C)** and 8 weeks **(E, F, G)** after unilateral injection into the masseter. Quantification of cartilage thickness, 4 weeks **(D)** and 8 weeks **(H)** after injection. Histograms **(D-H)** represent means ± SD for n = 8 per group. Significant difference between groups (*p < 0.05, **p<0.05, ***p<0.005, ****p<0.0001). Scale bar = 100µm.

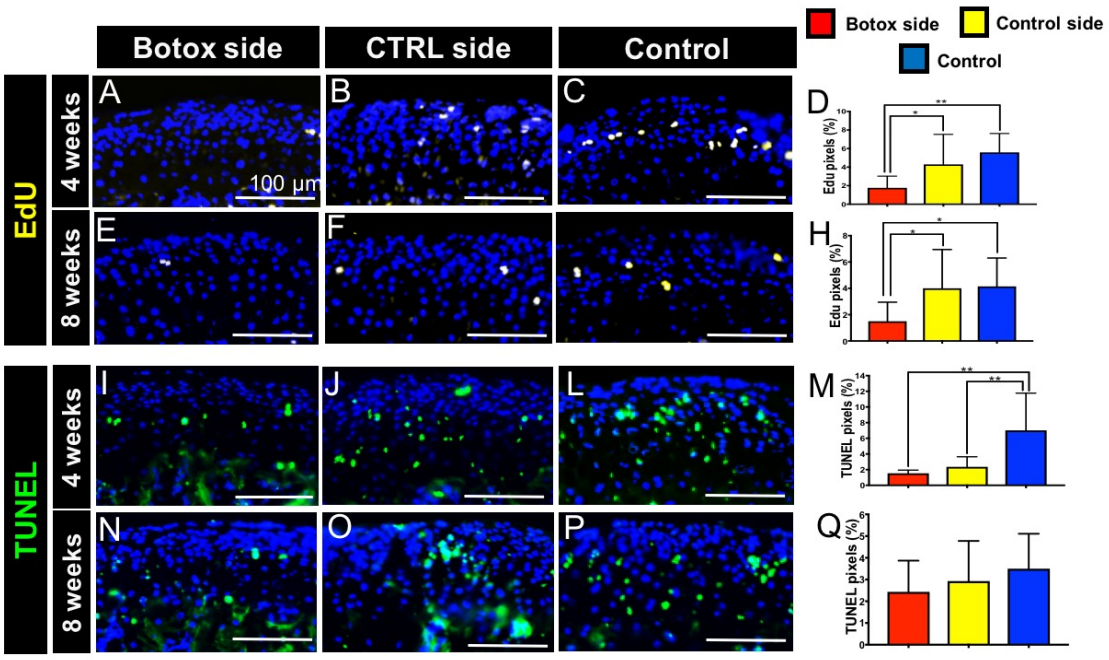

Figure 5: Reduced cell proliferation and cell apoptosis at the mandibular condylar cartilage of the Botox injected side, 4 and 8 weeks after injection into the masseter. Sagittal sections of condyles of Botox injected **(A, E),** control side **(B, F)** and control mice **(C, G)** stained for EdU. Quantification of EdU positive pixels (yellow) over DAPI positive pixels (blue) at the proliferative zone, 4 weeks **(D)** and 8 weeks **(H)** after injection. TUNEL staining in sections of Botox injected side **(I, N),** control side **(J, O)** and control mice **(L, P)** condyles. Quantification of TUNEL positive pixels (green) over DAPI positive pixels (blue) at the MCC, 4 weeks **(M)** and 8 weeks **(Q)** after injection. Histograms **(D, H, M, Q)** represent means ± SD for n = 8 per group. Significant difference between groups (*$p < 0.05$, **$p<0.05$). Scale bar = 100µm.

	4 weeks after injection				8 weeks after injection			
	A. Botox side	B. Control side	C. Pure Control	P value	A. Botox side	B. Control side	C. Pure Control	P value
BVF (%)	59.95 SD 3.97	64.3 SD 2.88	66.78 SD 3.37	A vs B * B vs C - ns A vs C **	66.97 SD 3.18	72.85 SD 2.38	73.28 SD 4.65	A vs B * B vs C - ns A vs C *
TRAP (%)	2.77 SD 1.38	8.36 SD 5.13	6.80 SD 4.80	A vs B ** B vs C - ns A vs C *	2.91 SD 1.30	6.07 SD 2.27	7.13 SD 3.20	A vs B ** B vs C - ns A vs C ****
Calcein (%)	1.49 SD 0.97	8.54 SD 0.97	11.37 SD 3.40	A vs B ** B vs C - ns A vs C ***	4.62 SD 1.56	9.24 SD 4.32	15.31 SD 12.15	A vs B - ns B vs C - ns A vs C *
Alizarin (%)	1.47 SD 0.92	10.01 SD 5.01	10.51 SD 5.08	A vs B * B vs C - ns A vs C *	4.91 SD 1.40	12.76 SD 5.84	14.79 SD 9.86	A vs B * B vs C - ns A vs C **
Cartilage Thick.(µm)	117.3 SD 13.51	135.3 SD 11.47	133.7 SD 12.77	A vs B ** B vs C - ns A vs C ***	127.8 SD 6.14	144.00 SD 8.31	161.2 SD 7.31	A vs B ** B vs C *** A vs C ****
EdU (%)	1.76 SD 1.25	4.29 SD 3.2	5.58 SD 2.03	A vs B * B vs C - ns A vs C **	1.49 SD 1.44	2.94 SD 2.94	4.14 SD 2.15	A vs B * B vs C - ns A vs C *
TUNEL (%)	1.53 SD 0.40	2.35 SD 1.28	7.01 SD 4.74	A vs B - ns B vs C ** A vs C **	2.09 SD 1.08	2.92 SD 1.84	3.49 SD 1.6	A vs B - ns B vs C - ns A vs C - ns

Table 1: Quantification data for bone volume fraction (BVF), trap, calcein, alizarin, cartilage thickness, Edu and Tunel. N = 8 per group. Significant difference between groups (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$). SD – stardand deviation, ns – not significant.