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

Eliane H. Dutra and Sumit Yadav
Farmington, Conn

Objectives: To evaluate whether the effects on the mandibular condylar cartilage (MCC) and subchondral bone are transient of botulinum neurotoxin (Botox) injection into the masseter muscle. **Methods:** Botox (0.3 U) was injected into the right masseter of 6-week-old female mice (C57BL/6; n = 16). In addition, 16 mice were used as control and received no injections. Experimental and matching control mice were killed 4 or 8 weeks after the single Botox injection. Mandibles and mandibular condyles were analyzed by means of microscopic computed tomography (microCT) and histology. Sagittal sections of condyles were stained for tartrate-resistant acid phosphatase (TRAP), toluidine blue, 5-ethynyl-2′-deoxyuridine (EdU), and terminal deoxynucleotide transferase–mediated dUTP nick-end labeling. **Results:** Bone volume fraction was significantly decreased on the subchondral bone of the Botox-injected side, compared with the control side and control mice, 4 and 8 weeks after injection. Furthermore, histologic 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. **Conclusions:** The effects on the MCC and subchondral bone of Botox injection into the masseter muscle persisted for 8 weeks after injection and were not considered to be transient. (Am J Orthod Dentofacial Orthop 2019;156:193-202)

The expanded classification of temporomandibular joint disorders (TMDs) includes temporomandibular joint (TMJ) conditions, masticatory muscle disorders, headaches, and abnormalities of related structures, such as coronoid hyperplasia.[1] Affected individuals suffer from functional and psychologic 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 neuromuscular 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 on the mandibular condyle; nonhuman animal as well as clinical studies have reported decreases 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 nonmineralized 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

Division of Orthodontics, University of Connecticut Health Center, Farmington, Conn.
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Address correspondence to: Sumit Yadav, 263 Farmington Avenue, L7063 MC1725, University of Connecticut Health Center, Farmington, CT 06030; e-mail, yadav_sumit17@yahoo.com.
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cartilage of the TMJ. In addition, it remains to be determined if the MCC would adjust to the altered loading pattern induced by Botox injection into the masseter. The aims of the present study were (1) to evaluate the long-term effects of 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 would be different and that short-term effects on the mandibular condyle are transient and would be diminished over time.

MATERIAL AND METHODS

The Institutional Animal Care Committee of the University of Connecticut Health Center reviewed and approved all procedures. The experiments followed the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.[17] We used 6-week-old female mice (C57BL/6; n = 32) obtained from Jackson Laboratories (Bar Harbor, Me). Mice at 6 weeks of age are considered to be in late puberty stage, and at 10 and 14 weeks (corresponding to the time points evaluated) are in adulthood (early 20 years of age if compared to humans).[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.

Botox (Onabotulinum toxin A; Allergan, Parsippany-Troy Hills, NJ) was injected into the right-side masseter of the experimental mice (0.3 U, 30 μL). The left-side masseter of experimental mice did not receive any injection, and the left condyle was considered to be the control side. Mice were anesthetized with the use of ketamine (90 mg/kg) and xylazine (13 mg/kg) before the injections.

We used 2 different end points to evaluate the short-term and long-term effects of Botox injection into the masseter muscle: short term (n = 8), mice were killed 4 weeks after unilateral Botox injection into the masseter; long-term (n = 8), animals were killed 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 in 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 they were killed. Moreover, mice were injected with the cell proliferation marker 5-ethynyl-2′-deoxyuridine (EdU; Life Technologies, Grand Island, NY), in a concentration of 30 mg/kg body weight 48 and 24 hours before they were killed. Mice were killed at each time point by means of CO_2 asphyxiation, and death was assured by means of cervical dislocation.

Before dissecting the mandibles, facial tissues around the masseter and mandible were removed, exposing the masseters. Photographs of the dissected mice were take with the use of a digital camera (Canon EOS Rebel T3i).

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). Frozen sagittal sections of the condyles (5 μm) were made with the use of the Kawamoto method.[19]

We used microscopic computed tomography (Scanco Medical, Brüttisellen, Switzerland) to analyze the bone volume fraction (BVF; %) of the mineralized cartilage and subchondral bone of the condyles. 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 16 mm holder. Serial tomographic projections were acquired at 55 kV and 145 μA, with a voxel size of 6 μm and 1000 projections per rotation collected at 300,000 μs. The DICOM images were transferred, segmented, and reconstructed with the use of the Mimics software (Materialise, Leuven, Belgium). To distinguish calcified tissue from noncalcified tissue, an automated algorithm using local threshold segmented the reconstructed grayscale images.

Slides were initially scanned with the use of a fluorescent microscope (Axio Observer; Carl Zeiss, Thornwood, NY). Next, the coverslip was removed by soaking the slides in phosphate-buffered saline solution and the same sections were stained for tartrate-resistant acid phosphatase (TRAP) with the use of ELF97 (Life Technologies), generating a yellow fluorescent signal. After imaging for TRAP, sections were stained for EdU (ClickiT EdU Alexa Fluor 555 HCS kit; Life Technologies) and imaged. Finally, the same slide was stained for toluidine blue and reimaged. Different slides were also stained for terminal deoxynucleotide transferase–mediated dUTP nick-end labeling (TUNEL; Deadend Fluorometric TUNEL System; Promega, Madison, Wis).

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

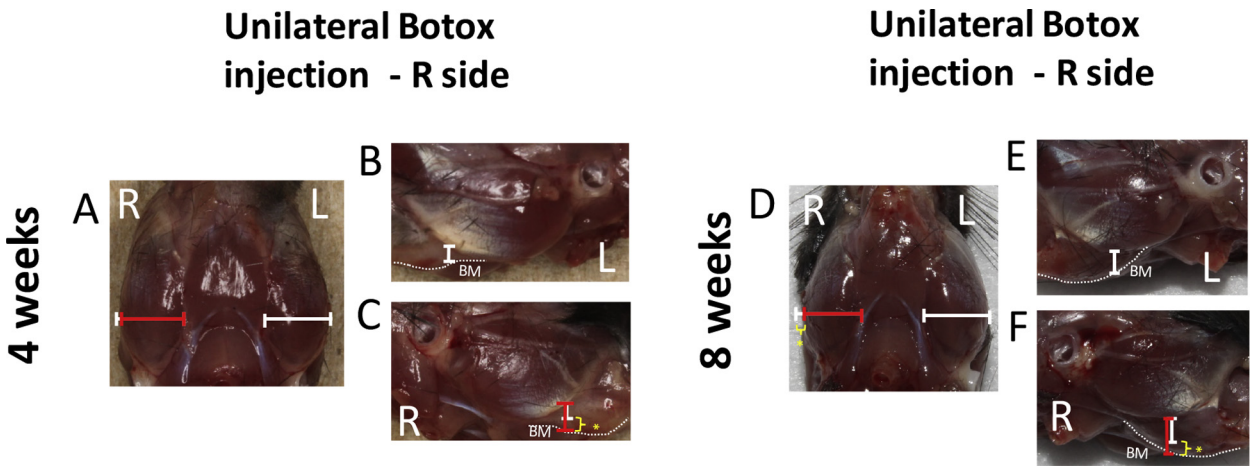

Fig 1. Changes in volume of masseter 4 and 8 weeks after Botox injection. **A,** Photograph of a dissected experimental mouse 4 weeks after unilateral injection on the right (R) side observed from the inferior view. The white bar represents the lateral-medial length of the contralateral side (L), and the red bar the length on the injected side (R). The difference between the 2 sides is represented by overlapping the 2 bars on the R side. The right-side masseter is slightly smaller on the injected side (R) compared with contralateral 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 shown. **B,** The white bar represents the contralateral side and **C,** the red bar the injected side. There is a much larger distance on the injected side (R) compared with the contralateral side (L), suggesting that the masseter was shorter on the Botox-injected side. **D,** Photograph of a dissected experimental mouse 8 weeks after unilateral injection on the right (R) side observed from the inferior view. The difference between the 2 sides is represented by overlapping the 2 bars at the R side. The right-side masseter is substantially smaller on the injected side (R) compared with the contralateral 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 shown. **E,** The white bar represents the contralateral side and **F,** the red bar the injected side. Similarly to what was observed after 4 weeks, the distance on the injected side (R) was much larger than on the contralateral side (L), suggesting that the masseter was still shorter on the Botox injected side after 8 weeks.

staining and fluorochrome labels was performed with the use of Adobe Photoshop (Adobe Systems, San Jose, Calif). 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 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-positive pixels (green) over DAPI-positive pixels (blue) in the MCC.

Cartilage thickness (Fig 4) was analyzed with the use of Digimizer Image software (MedCalc Software, Ostend, Belgium); measurements were performed from the outer cellular layer of MCC to the tidemark (in 5 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 BVF and histologic analysis. Outcomes were compared between the Botox-injected side, control side, and matching pure control groups. Statistically significant differences among means were determined by means of 1-way analysis of variance. A P value of <0.05 was deemed to be statistically significant (Table). Statistical analyses were computed with the use of GraphPad Prism (GraphPad Software, La Jolla, Calif).

RESULTS

Botox injections into the masseter are also used as a cosmetic procedure to reduce the thickness of the

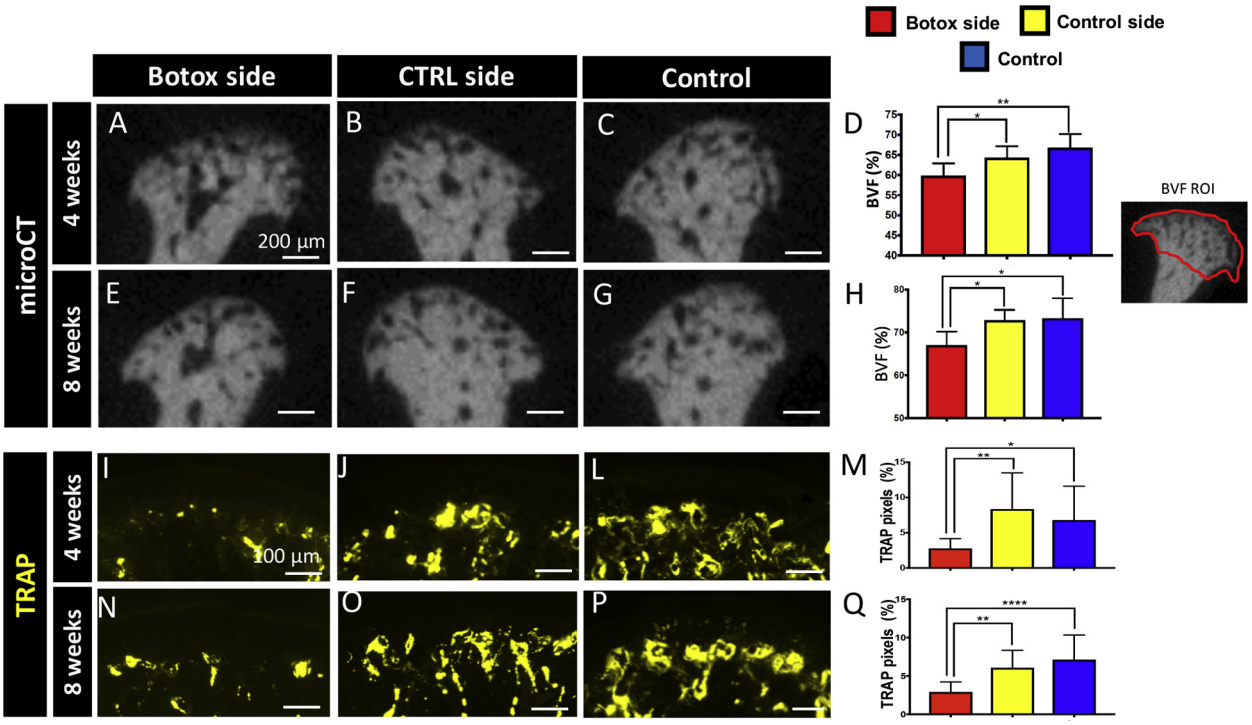

Fig 2. Reduced bone volume and bone remodeling of the condyle on the Botox-injected side 4 and 8 weeks after injection into the masseter. Coronal micro-CT images of condyles on **A, E,** Botox-injected side, **B, F,** control side, and **C, G,** pure control mice **A-C,** 4 weeks ands **E-G,** 8 weeks 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 (*yellow*): **I, N,** Botox-injected side, **J, O,** control side, and **L, P,** pure control mice **I, J, L,** 4 weeks and **N-P,** 8 weeks after unilateral injection. **M,** Quantification of TRAP-positive pixels over subchondral bone area 4 weeks after injection. **Q,** Quantification of TRAP-positive pixels 8 weeks after injection. Histograms **(D, H, M, Q)** represent mean ± SD for n = 8 per group. Significant differences between groups: $*P < 0.05$; $**P < 0.05$; $****P < 0.0001$. Scale bars: **A-G,** 200 µm, **I-P,** 100 µm. *BVF ROI*, bone volume fraction region of interest.

masseter.[20] 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 and 8 weeks after injection. We noticed a mild reduction in masseter volume on the Botox-injected side (right side) compared with the contralateral side 4 weeks after unilateral injection (Fig 1, *A*), but a more prominent reduction in muscle size 8 weeks after unilateral injection (Fig 1, *B*).

We used microCT analysis to compare the bone volume of the mineralized cartilage and the subchondral bone between the 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 the experimental mice 4 and 8 weeks after injection. We observed a 10% decrease in the BVF in the Botox-injected side compared with control side and control mice 4 weeks after injection (Fig 2, *A-D*; Table). The observed reduction in BFV persisted for 8 weeks after injection (Fig 2, *E-H*; Table). 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 compared with control side and pure control, as revealed by ∼60% decrease in TRAP-positive pixels in the Botox side compared with controls 4 weeks after injection (Fig 2, *I-M*; Table). Likewise, there was ∼50% reduction in TRAP activity 8 weeks after unilateral injection (Fig 2, *N-Q*; Table). Next, we analyzed the fluorochrome labels calcein and alizarin complexone. Our quantification revealed a substantial reduction of 80% in calcein labeling (Fig 3, *A-C* and *G*; Table) and 90% decrease in alizarin complexone (Fig 3, *A-C* and *H*, Table) in the subchondral region 4 weeks after Botox

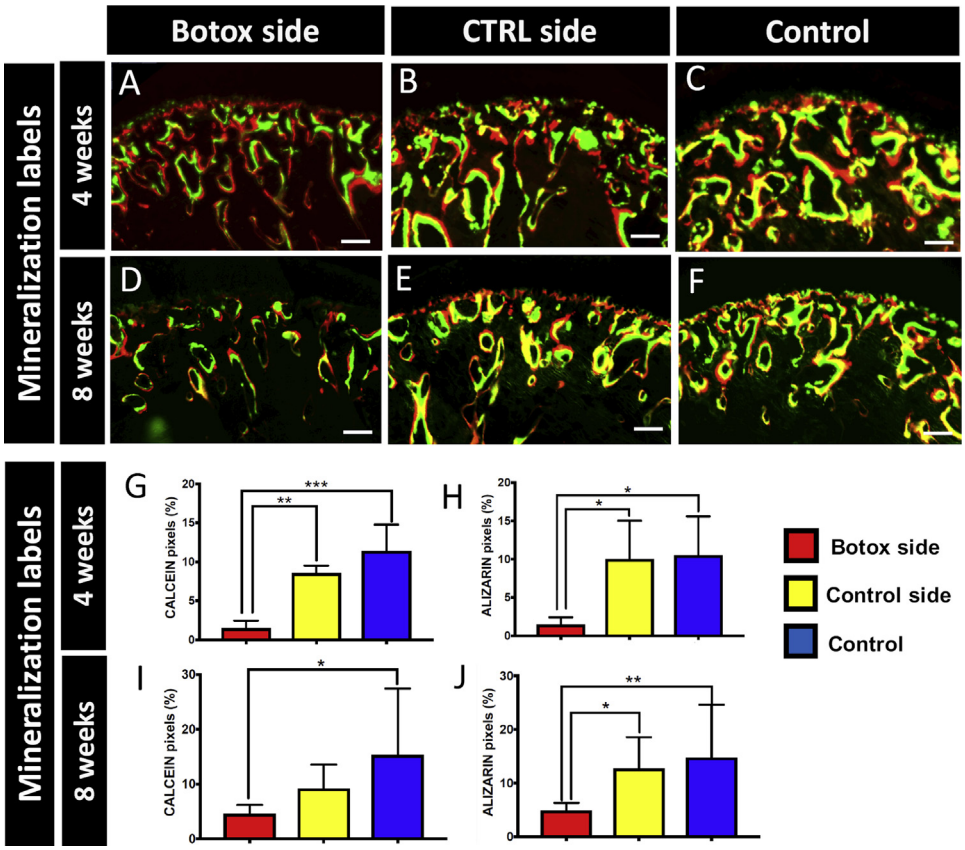

Fig 3. Decreased mineralization in the subchondral region on the Botox-injected side 4 and 8 weeks after injection into the masseter. Sagittal sections of condyles of **A, D,** Botox-injected side, **B, E,** control side, and **C, F,** pure control mice **A-C,** 4 weeks and **D-F,** 8 weeks after unilateral injection into the masseter. Quantification of fluorochrome label–positive pixels (*green:* calcein; *red:* alizarin complexone) 4 and 8 weeks after injection: **G,** calcein after 4 weeks, **H,** alizarin complexone after 4 weeks, **I)** calcein after 8 weeks, **J)** alizarin complexone after 8 weeks. Histograms **(G-J)** represent mean ± SD for n = 8 per group. Significant differences between groups: $^{*}P < 0.05$; $^{**}P < 0.05$; $^{***}P < 0.005$. Scale bars = 75 μm.

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 in the Botox-injected side compared with control side, and a 70% reduction when the Botox-injected side was compared with the pure control, 8 weeks after injection (Fig 3, *D-F* and *I*; Table). Similarly, alizarin complexone labeling was ∼65% decreased 8 weeks after injection (Fig 3, *D-F* and *J*; Table).

We next evaluated the structural and cellular changes within the MCC 4 and 8 weeks after unilateral Botox injection into the masseter. We started by analyzing the cartilage thickness by means of toluidine blue staining. Quantification of the toluidine blue distance mapping showed a significant reduction in cartilage thickness in the Botox-injected side compared with control side and pure control groups 4 weeks after injection (Fig 4, *A-D*; Table). Interestingly, cartilage thickness analysis 8 weeks after injection suggested that not only the Botox-injected side, but also the contralateral (control side), was affected (Fig 4, *E-H*; Table).

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 5, *A-H*; Table). Subsequently, cell apoptosis was evaluated by means of TUNEL staining. In all groups, TUNEL-positive cells were observed, mostly at the prehypertrophic layer of the MCC (Fig 5, *I-L* and *N-P*). Surprisingly, there was no statistically significant difference between Botox side and control side, but there were significantly fewer TUNEL-positive cells in the MCC of these groups

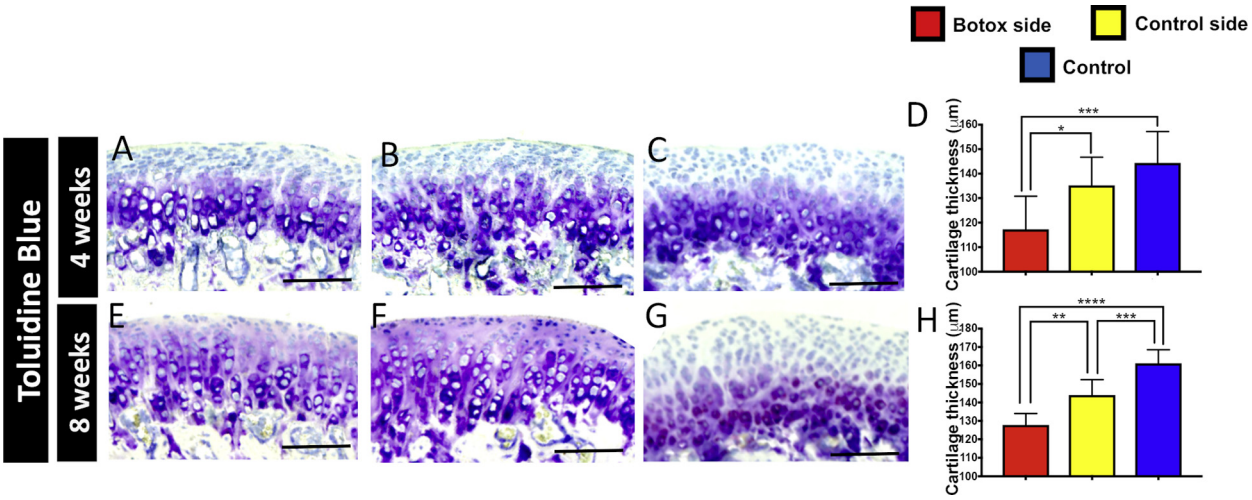

Fig 4. Reduced cartilage thickness of the mandibular condyle on the Botox-injected side 4 and 8 weeks after injection into the masseter. Sagittal sections of condyles stained for toluidine blue: **A, E,** Botox-injected side, **B, F,** control side, and **C, G,** pure control mice **A-C,** 4 weeks and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

compared with pure control 4 weeks after injection (Fig 5, *A-D*; Table). 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 on cell apoptosis were reversed 8 weeks after injection (Fig 5, *N-Q*; Table).

DISCUSSION

Contrary to our hypothesis, the effects of Botox were 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 6-week-old mice on the MCC and subchondral bone. We found that most of the effects observed 4 weeks after 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.[5,22] Furthermore, the duration of muscular paralysis seems to be dependent on the dosage of the Botox injection.[23] Our dosage of 0.3 U into the masseter muscle was within the range of low dose for rodents.[24]

The long-term effect of Botox injections into the masseter of the rabbits has been studied by Rafferty et al,[9] who found decreased bone volume of the subchondral bone on 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 that 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 evidenced by a reduction in osteoclast activity and osteoblastic activity (mineral deposition), represented by a significant reduction in the number of osteoclasts and in 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

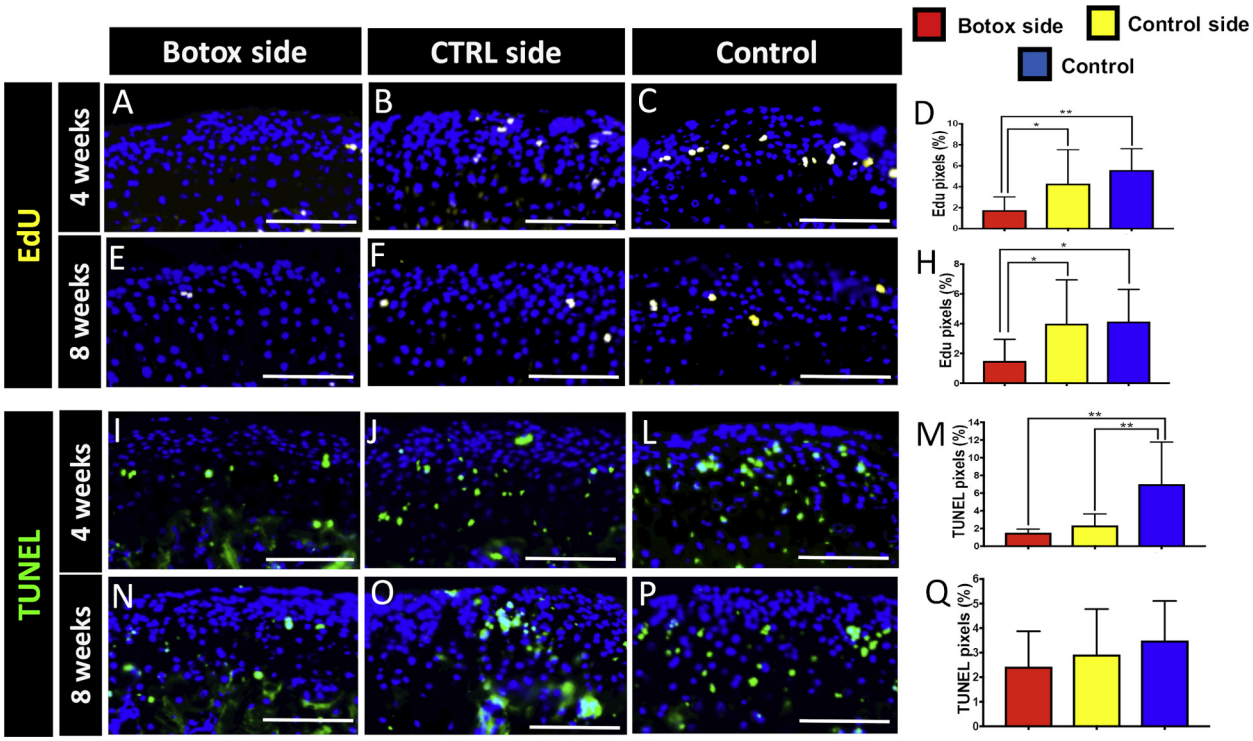

Fig 5. Reduced cell proliferation and cell apoptosis in the mandibular condylar cartilage of the Botox-injected side 4 and 8 weeks after injection into the masseter. Sagittal sections of condyles of **A, E,** Botox-injected side, **B, F,** control side, and **C, G,** control mice stained for EdU. Quantification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

increase in osteoclastogenesis, which may have occurred within a few days after Botox injection. Aliprantis et al[25] 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 compared with 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 after injection, leading to an increase in osteoclast fusion and expression of pro-osteoclastic genes 72 hours after paralysis.[26] In addition, it has been shown that the bone loss in murine models due to muscle atrophy is due to both decreased osteoblastic activity and increased osteoclastic activity.[27] 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 nontransiently to acute muscular paralysis.

We observed a reduction in cartilage thickness, initially noticed only on the injected side at 4 weeks but at both injected and contralateral sides at 8 weeks, suggesting that both sides are affected and altered in loading 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, but no effects on the cartilage thickness were noticed as a result of treatment.[28] 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,

Table. Quantification data for bone volume fraction (BVF), trap, calcein, alizarin, cartilage thickness, Edu, and TUNEL

	4 weeks after injection				*8 weeks after injection*			
Measure	*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 complexone (%)	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 thickness (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)

n = 8 per group. Significant differences between groups: *P <0.05; †P <0.005; ‡P <0.0005; §P <0.0001.

but also a diminished cartilage width as a consequence of masseter paralysis.

The MCC is formed by 4 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 composed of mature chondrocytes, cells that still have the potential to proliferate. The fourth and deepest layer is formed by hypertrophic chondrocytes, which die, have their cytoplasm evacuated, and undergo mineralization.[14,29-31] Our EdU proliferation assay showed significantly decreased cell proliferation in the MCC of the injected side, which were consistent with the effects seen after TMJ unloading experiments in mice,[13,14] 4 and 8 weeks after injection. The nontransient reduction in cell proliferation suggests that the unloading effect in the MCC likely persists for the period when 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 on 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 with the use of cone-beam computed tomography the condyles of women who received at least 1 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 of the 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 1 year.[32]

One of the limitations of the present 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 on the mandibular condyle of mice, as

presented here, suggests that the MCC does not adapt to the change 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 in the MCC at 4 weeks and 8 weeks after Botox injection into the masseter muscle.

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REFERENCES

1. Peck CC, Goulet JP, Lobbezoo F, Schiffman EL, Alstergren P, Anderson GC, et al. Expanding the taxonomy of the diagnostic criteria for temporomandibular disorders. J Oral Rehabil 2014; 41:2-23.
2. Connelly ST, Myung J, Gupta R, Tartaglia GM, Gizdulich A, Yang J, et al. Clinical outcomes of Botox injections for chronic temporomandibular disorders: do we understand how Botox works on muscle, pain, and the brain? Int J Oral Maxillofac Surg 2017;46: 322-7.
3. Pihut M, Ferendiuk E, Szewczyk M, Kasprzyk K, Wieckiewicz M. The efficiency of botulinum toxin type A for the treatment of masseter muscle pain in patients with temporomandibular joint dysfunction and tension-type headache. J Headache Pain 2016; 17:29.
4. Ivask O, Leibur E, Akermann S, Tamme T, Voog-Oras U. Intramuscular botulinum toxin injection additional to arthrocentesis in the management of temporomandibular joint pain. Oral Surg Oral Med Oral Pathol Oral Radiol 2016;122:e99-106.
5. de Paiva A, Meunier FA, Molgo J, Aoki KR, Dolly JO. Functional repair of motor endplates after botulinum neurotoxin type A poisoning: biphasic switch of synaptic activity between nerve sprouts and their parent terminals. Proc Natl Acad Sci U S A 1999;96:3200-5.
6. Kim JY, Kim ST, Cho SW, Jung HS, Park KT, Son HK. Growth effects of botulinum toxin type A injected into masseter muscle on a developing rat mandible. Oral Dis 2008;14:626-32.
7. Tsai CY, Huang RY, Lee CM, Hsiao WT, Yang LY. Morphologic and bony structural changes in the mandible after a unilateral injection of botulinum neurotoxin in adult rats. J Oral Maxillofac Surg 2010;68:1081-7.
8. Tsai CY, Yang LY, Chen KT, Chiu WC. The influence of masticatory hypofunction on developing rat craniofacial structure. Int J Oral Maxillofac Surg 2010;39:593-8.
9. Rafferty KL, Liu ZJ, Ye W, Navarrete AL, Nguyen TT, Salamati A, et al. Botulinum toxin in masticatory muscles: short- and long-term effects on muscle, bone, and craniofacial function in adult rabbits. Bone 2012;50:651-62.
10. Raphael KG, Tadinada A, Bradshaw JM, Janal MN, Sirois DA, Chan KC, et al. Osteopenic consequences of botulinum toxin injections in the masticatory muscles: a pilot study. J Oral Rehabil 2014;41:555-63.
11. Dutra EH, O' Brien MH, Lima A, Kalajzic Z, Tadinada A, Nanda R, et al. Cellular and matrix response of the mandibular condylar cartilage to botulinum toxin. PLoS One 2016;11:e0164599.
12. Utreja A, Dyment NA, Yadav S, Villa MM, Li Y, Jiang X, et al. Cell and matrix response of temporomandibular cartilage to mechanical loading. Osteoarthritis Cartilage 2016;24:335-44.
13. Chen J, Sorensen KP, Gupta T, Kilts T, Young M, Wadhwa S. Altered functional loading causes differential effects in the subchondral bone and condylar cartilage in the temporomandibular joint from young mice. Osteoarthritis Cartilage 2009;17: 354-61.
14. Shen G, Darendeliler MA. The adaptive remodeling of condylar cartilage—a transition from chondrogenesis to osteogenesis. J Dent Res 2005;84:691-9.
15. Benjamin M, Ralphs JR. Biology of fibrocartilage cells. Int Rev Cytol 2004;233:1-45.
16. Foran PG, Mohammed N, Lisk GO, Nagwaney S, Lawrence GW, Johnson E, et al. Evaluation of the therapeutic usefulness of botulinum neurotoxin B, C1, E, and F compared with the long lasting type A: basis for distinct durations of inhibition of exocytosis in central neurons. J Biol Chem 2003;278:1363-71.
17. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. PLoS Biol 2010;8:e1000412.
18. Dutta S, Sengupta P. Men and mice: relating their ages. Life Sci 2016;152:244-8.
19. Kawamoto T. Use of a new adhesive film for the preparation of multi-purpose fresh-frozen sections from hard tissues, whole-animals, insects and plants. Arch Histol Cytol 2003;66:123-43.
20. Yu CC, Chen PK, Chen YR. Botulinum toxin a for lower facial contouring: a prospective study. Aesthetic Plast Surg 2007;31: 445-51: discussion 452-443.
21. Dimitrova DM, Shall MS, Goldberg SJ. Short-term effects of botulinum toxin on the lateral rectus muscle of the cat. Exp Brain Res 2002;147:449-55.
22. Jurasinski CV, Lieth E, Dang Do AN, Schengrund CL. Correlation of cleavage of SNAP-25 with muscle function in a rat model of botulinum neurotoxin type A induced paralysis. Toxicon 2001;39: 1309-15.
23. Washbourne P, Pellizzari R, Rossetto O, Bortoletto N, Tugnoli V, de Grandis D, et al. On the action of botulinum neurotoxins A and E at cholinergic terminals. Journal of Physiology-Paris 1998;92:135-9.
24. Chappard D, Chennebault A, Moreau M, Legrand E, Audran M, Basle MF. Texture analysis of x-ray radiographs is a more reliable descriptor of bone loss than mineral content in a rat model of localized disuse induced by the *Clostridium botulinum* toxin. Bone 2001;28:72-9.
25. Aliprantis AO, Stolina M, Kostenuik PJ, Poliachik SL, Warner SE, Bain SD, et al. Transient muscle paralysis degrades bone via rapid osteoclastogenesis. FASEB J 2012;26:1110-8.
26. Ausk BJ, Worton LE, Smigiel KS, Kwon RY, Bain SD, Srinivasan S, et al. Muscle paralysis induces bone marrow inflammation and predisposition to formation of giant osteoclasts. Am J Physiol Cell Physiol 2017;313:C533-40.
27. Sakata T, Sakai A, Tsurukami H, Okimoto N, Okazaki Y, Ikeda S, et al. Trabecular bone turnover and bone marrow cell development in tail-suspended mice. J Bone Miner Res 1999;14:1596-604.
28. Matthys T, Ho Dang HA, Rafferty KL, Herring SW. Bone and cartilage changes in rabbit mandibular condyles after 1 injection of botulinum toxin. Am J Orthod Dentofacial Orthop 2015;148: 999-1009.

29. Wadhwa S, Kapila S. TMJ disorders: future innovations in diagnostics and therapeutics. J Dent Educ 2008;72:930-47.
30. Ohno S, Schmid T, Tanne Y, Kamiya T, Honda K, Ohno-Nakahara M, et al. Expression of superficial zone protein in mandibular condyle cartilage. Osteoarthritis Cartilage 2006;14: 807-13.
31. Shibukawa Y, Young B, Wu C, Yamada S, Long F, Pacifici M, et al. Temporomandibular joint formation and condyle growth require Indian hedgehog signaling. Dev Dyn 2007;236:426-34.
32. Aziz J, Awal D, Ayliffe P. Resorption of the mandibular condyle after injections of botulinum toxin A. Br J Oral Maxillofac Surg 2017;55:987-8.