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Bone and cartilage changes in rabbit mandibular condyles after a single injection of botulinum toxin

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Abstract

Introduction—Temporary paralysis of the masseter muscle using botulinum toxin is a common treatment for temporomandibular disorders, bruxism, and muscle hypertrophy. Loss of masseter force is associated with decreased mandibular mineral density. Our objectives were (1) to establish whether bone loss at the mandibular condyle is regionally specific, and (2) to ascertain whether the treatment affects the condylar cartilage.

Methods—Young adult female rabbits received a unilateral masseter injection of botulinum neurotoxin serotype A (BoNT/A, n=31), saline (n=19) or no injection (n=3) and were also injected with bromodeoxyuridine (BrdU), a replication marker. Termination occurred 4 or 12 weeks following treatment. Condyles were processed by paraffin histology. Cortical thickness, cartilage thickness and trabecular bone areal density were measured, and replicating cells were counted after BrdU reaction.

Results—BoNT/A rabbits exhibited a high frequency of defects in the condylar bone surface, occurring equally on injected and uninjected sides. Bone loss was seen only on the side of the BoNT/A injection. Cortical as well as trabecular bone was severely affected. The midcondylar region lost the most bone. Recovery at 12 weeks was insignificant. Condylar cartilage thickness showed no treatment effect but did increase with time. Numbers of proliferating cells were similar in treatment groups, but BoNT/A animals showed more side asymmetry in association with the condylar defects.

Conclusion—Bone loss may be a risk factor for the use of botulinum toxin in jaw muscles.

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Author contributions:

Dr. Matthys performed all the condylar morphometry and analysis; Dr. Ho Dang performed all the histochemistry and the analysis of proliferation; both Drs. Matthys and Ho Dang independently performed the subjective analysis; Dr. Rafferty helped Drs. Matthys and Ho Dang with their analyses and compiled the first draft of the paper from Master's Theses completed by Drs. Matthys and Ho Dang; Dr. Herring originated and supervised the study and wrote the final draft of the paper.

INTRODUCTION

Botulinum neurotoxin causes paralysis of neuromuscular junctions by blocking the release of acetylcholine. Of the seven serotypes produced by *Clostridium* bacteria, botulinum neurotoxin type A (BoNT/A) is the most widely used by clinicians.¹ Both striated and smooth muscle respond to BoNT/A, and the approved applications range from erasing wrinkles to severe neurological movement disorders. Indeed, there are few parts of the body that have not become targets of BoNT/A.

Current off-label applications of BoNT/A include masseter muscle injection to reduce activity or to cause atrophy for cosmetic reasons. This therapy is often employed for alleviating pain in patients with temporomandibular disorders^{2,3} and related conditions^{4,5} as well as for neuromuscular diseases.⁶ In addition, BoNT/A is a popular cosmetic treatment of benign masseter hypertrophy^{7,8} despite the absence of controlled trials assessing the efficacy and safety of this procedure.⁹

The paralysis of a major jaw adductor muscle may have unintended consequences for the temporomandibular joint (TMJ). Mechanical loading is important for bone maintenance and likewise, underloading is generally detrimental to bone health. BoNT/A treatment of the masseter reduces muscle force and indeed has been used for that purpose after condylar fracture.^{10,11} However, loss of muscle force typically leads to rapid loss of bone mass in the mandible¹² as well as in limb bones.^{13,14} Radiologically, the mandibular condyle is strongly affected by administration of BoNT/A to the masseter. Both a rabbit study¹⁵ and a pilot study on TMJ patients¹⁶ indicated severe loss of bone density compared to controls. The rabbit study included microCT scans of condyles 4 weeks and 12 weeks after unilateral masseter injection. Central slices indicated that the injection-side condylar head had lost 40% bone area at 4 weeks relative to the uninjected side and saline controls, and even at 12 weeks was 15% under control values.¹⁵ The present contribution is a histological follow-up to that study and uses some of the same rabbit specimens to address questions that could not be answered from the microCT scans.

First, we sought to learn whether bone loss was specific to particular regions. Reasoning that the subchondral region receives loads directly from the overlying cartilage and transmits them to more distant condylar sites, we hypothesized that the subchondral trabeculae would show greater bone loss than more inferior trabeculae and the condylar cortex.

Second, we wanted to establish whether the condylar cartilage was affected as well as the bone. Cartilage is strongly affected by its mechanical environment; both understimulation and overstimulation can induce degenerative changes.¹⁷ Feeding rodents or rabbits a soft diet is claimed to thin the condylar cartilage^{18–20} although the tissue remains healthy.^{21–23} A single study using BoNT/A in the masseter of young rats (apparently without incisor clipping) reported thinner cartilage in association with increased apoptosis.²⁴ We expected that reduced loading of the TMJ caused by BoNT/A masseter injection would be analogous to a soft diet, and thus there would be decreased proliferation and reduced thickness of the condylar cartilage after BoNT/A injection of the masseter.

MATERIALS AND METHODS

Sample

All procedures were approved by the Institutional Animal Care and Use Committee of the xxx. While under isoflurane anesthesia, 50 female New Zealand rabbits, 5 months old at acquisition, received unilateral injections of either 10U of BoNT/A (n=31, Botox™, Allergan Inc., Irvine, CA) or an equivalent volume of 0.9% saline (n=19) to the inferior portion of the superficial masseter muscle (split among three separate injection points¹⁵); the contralateral uninjected side served as an intra-animal control. Most of the animals were derived from the previous study.¹⁵ These animals were terminated 4 weeks (“atrophy”) or 12 weeks (“recovery”) after injection, therefore establishing four test groups: BoNT/A 4 week, BoNT/A 12 week, saline 4 week, and saline 12 week. Sample sizes for analysis (Tables 1–4) varied from 7 to 13 per group because of sectioning or staining errors. Three additional rabbits were untreated controls and were terminated 1–2 weeks after acquisition.

One week prior to euthanasia, each animal was anesthetized and injected i.v. with bromodeoxyuridine (BrdU), a thymidine analog that labels cells in the S-phase of replication (40 mg/kg as a 10 mg/ml solution in phosphate-buffered saline). On the day of the assigned endpoint, the animals were perfused with 0.9% phosphate buffered saline followed by 4% paraformaldehyde. The mandibular condyles of each rabbit were removed from the mandibular ramus, decalcified in Immunocal™ (Decal Chemical Corp., Tallman, NY), embedded in paraffin, and sectioned in the coronal plane at 7–10 µm. The observers (TM for condylar measurements and HH for BrdU labeled cell counts and subjective observations) were blinded to the treatment of the specimens.

Condylar observations and morphometrics

Three coronal sections through the widest portion of each condyle were stained with hematoxylin and eosin. Damaged sections were discarded, resulting in a few cases where only one or two sections were used. Our original intent was to follow the standard staining and BrdU reaction with immunohistochemical stains for osteoblastic and osteoclastic markers. However, due to problems with decalcification and infiltration leading to the necessity of re-embedding many specimens, the cellular contents of many marrow cavities (where osteoblastic and osteoclastic cells would have been found) were disconnected from bone surfaces, which caused distortion and in some cases missing marrow contents. Thus we could not reliably identify either osteoblasts or osteoclasts in the marrow cavities. Instead, each section was just qualitatively examined for signs of atypical morphology (unusual shape, surface defects, etc.) and of resorption (multinucleated osteoclasts in Howship’s lacunae or scalloped surface). Then the section was aligned along the long axis of the condylar process and imaged at 10× (Nikon E400 microscope) using MetaVue (Molecular Devices LLC, Sunnyvale, CA). Measurements were made using MetaMorph (Molecular Devices) and averaged for the three sections per condyle.

Measurements are illustrated in Fig. 1. Thickness of the condylar cartilage was measured at the most apical portion of the articular surface, and included all zones (fibrous, proliferative, maturational and hypertrophic), as separate measurements of each zone were not adequately

repeatable. Percent trabecular bone area (areal density) was assessed by superimposing a standardized grid over the chosen regions and counting the number of intersections that fell on bone; this number was divided by the total number of grid intersections to yield a proportion. The two chosen regions were subchondral and midcondylar (for details, see Fig. 1 caption). Cortical thickness was measured on medial and lateral sides of the condylar neck perpendicular to the long axis of the cortex at a standard distance from the midcondylar region.

Cell replication

An additional five sections were chosen from the central region of each condyle; one slide was used as a negative control and four were reacted for BrdU using a kit and following the manufacturer's instructions (Becton Dickinson, Franklin Lakes, NJ). Sections were lightly counterstained with methyl green. A brown nucleus was the defining feature for a BrdU-positive (replicating) cell. The cartilage was divided into thirds (medial, central, lateral) as shown in Fig. 1. Three superficial-to-deep zones were identified for counting BrdU-labeled cells: dense fibrous connective tissue, designated the "fibrous zone" (Fib Z, Fig. 2), small, flat cells underlying and parallel to the fibrous zone, designated "proliferative zone" (Pro z), and an area grading from slightly larger ovoid cells to large spherical hypertrophic chondrocytes designated "maturing and hypertrophic zone" (M&H Z). However, the boundaries of these zones were not distinct. In addition to the condylar cartilage counts, positive cells in the periosteum of the condylar poles and neck and in the lining of marrow cavities were noted. Counts from the 4 sections per condyle were averaged.

Statistical analysis

Measurement error for condylar morphometrics was assessed by remeasuring 10 randomly selected slides several months after the first measurement and calculating Dahlberg's formula.²⁵ Measurement error for BrdU-positive cell counts was assessed using this formula on 25 randomly selected slides.

Data were analyzed by comparing (1) the injected side with the opposite, uninjected side of the same animal, with paired tests; (2) the BoNT/A sample with the saline sample using 2-sample tests; and (3) analysis of covariance (ANCOVA). Except for the ANCOVA, cell counts were analyzed with nonparametric methods (Wilcoxon for paired tests, Mann-Whitney for 2-sample tests, and Friedman for related 3-sample tests); for morphometric data parametric methods (matched pair t-test for paired data, Student's t-test for 2-sample tests) were used. The ANCOVA utilized difference scores equal to the injected side minus the uninjected side and a regression model adjusted for the magnitude of the uninjected side measurement. Models that included the group by covariate interactions and the group by time interactions were also evaluated, but the significance of the interactions did not justify their inclusion in the final models. Analyses were conducted using Excel, SPSS and GraphPad QuickCalcs.

RESULTS

Functional observations

As reported previously,¹⁵ the rabbits showed no overt effects of the treatment, although bite force elicited by stimulating the injected masseter was greatly reduced. Body weight remained constant in all groups. There was no shift of mandibular position in BoNT/A-injected animals, which continued to chew on both sides of the jaw at the normal rate.

Measurement error

Measurement error ranged from 1.4% (subchondral percent trabecular bone area) to 6–7% (midcondylar percent trabecular bone area and cortical thickness). The higher values reflect the difficulty of repositioning the grids to the same locations but were still considered acceptable. Cartilage thickness and cell counts had intermediate measurement errors (2–5%).

Subjective observations of condylar morphology and resorption

32 of the 38 condyles from uninjected controls and saline-injected animals exhibited a fungiform shape with clearly defined poles in coronal section (Fig. 3A). The most apical region was usually off-center toward the medial side. The articular cartilage followed the contour of the condylar head with the thickest portion at the apex and tapering towards the poles. However, the remaining 6 control condyles were recorded as having unusual morphology, specifically a bony defect, typically in the central or medial third, filled in with a local expansion of the fibrous layer (Fig. 3B, Table 1). This defect was often accompanied by an irregular surface contour.

In the BoNT/A-injected rabbits, the bony defect was more prevalent, affecting 7/24 injected side condyles and 12/24 uninjected side condyles (Table 1). Three of these affected condyles exhibited inclusions in the thickened fibrous layer (Fig. 3C). Fisher's exact test indicated significant differences in the frequency of this defect ($p = 0.03$) between BoNT/A and the other rabbits (control and saline groups combined) for almost all comparisons (Table 1).

Evidence of extensive resorption was lacking in all condyles. With the exception of the condylar poles, which sometimes housed a few osteoclasts and resorption cavities, trabecular and periosteal bone surfaces were smooth and lacked Howship's lacunae. The few osteoclasts found in the interior appeared to be attacking remnants of cartilage buried in subchondral trabecular bone rather than remodeling the trabeculae. No differences were seen between BoNT/A and control condyles.

Condylar Morphometry

The saline sample showed no trend for differences in bone content between the injected and uninjected sides at either time-point, nor were there indications that age (12 weeks rather than 4 weeks post-injection) had any effect. Moreover, the uninjected contralateral side of BoNT/A rabbits was always similar in bone content to the condyles of saline animals (Table 2).

However, the BoNT/A-injected side condyles showed substantial reductions in bone content (Table 2 and Fig. 4). At 4 weeks the BoNT/A-injected side condyles had consistently less trabecular bone than the contralateral side or saline condyles. The midcondylar area showed a greater injected-side reduction of bone (26% vs. 41–46%, $p = 0.01$) than did the subchondral area (52% vs. 62–64%, $p < 0.01$). Average cortical thickness (medial and lateral summed for greater consistency) was reduced from 1000–1200 μm to 836 μm , but with statistical significance ($p = 0.03$) only for the comparison with saline.

At 12 weeks the trabecular bone in the midchondral region had not recovered (28.5% vs. 36–39%, $p = 0.01$). The subchondral trabecular region was also still low (55.5%) but comparisons to the uninjected side and saline injection (60–62%) failed to satisfy $p < 0.05$, due at least in part to high variation. Cortical thickness averaged 926 μm , also still low compared to the contralateral side (1099 μm , $p = 0.01$) and saline injection (1010 μm , $p = 0.09$).

In contrast to the bone parameters, cartilage thickness at the apex of the condyle showed no BoNT/A effect (Table 2) at either 4 weeks or 12 weeks post-injection.

ANCOVA results in Table 3 strongly support the significant BoNT /A bone loss in all regions (all $p = 0.001$) and show further that there were no differences between 4 and 12 weeks ($p = 0.42$ – 0.66). For cartilage thickness, ANCOVA confirmed the absence of a treatment effect, but interestingly, demonstrated an age effect (Table 3), specifically a thickening of the cartilage. Regardless of side or treatment group, condylar cartilage was 220–262 μm thick at the 4-week endpoint and 315–341 μm thick at the 12-week endpoint ($p = 0.005$).

BrdU labeled cells

Regardless of treatment group or side, the periosteal of the condylar poles were sparsely labeled, averaging 5–10 cells/pole, and the periosteum of the condylar neck had no replicating (BrdU-positive) cells. Labeled cells were very frequent lining the subchondral marrow cavities but were not quantified because not all marrow cavities retained their cellular contents. The fibrous zone of the condylar cartilage rarely showed replicating cells, averaging 1/condyle 4 weeks post-injection and 2/condyle 12 weeks post-injection. The cartilage itself was always labeled, with positive cells most common in the transition area between the proliferative and the mature-hypertrophic zones (Fig. 2). These two zones did not differ consistently, (Wilcoxon tests), nor were there consistent differences among the medial, central and lateral thirds (Friedman tests). Therefore, only total BrdU-positive cell counts for the condylar cartilage are presented in Table 4. Except for the uninjected side of the 12-week post-BoNT/A injected animals (mean 100, median 54), the mean (40–50) and median (18–40) counts were similar for both sides and both treatment groups.

However, the striking finding in Table 4 is the large variation. Although some of this variation was interindividual, with some animals showing high or low levels of BrdU incorporation on both sides, many animals were surprisingly asymmetrical in labeling of the condylar cartilage. A comparison of side differences is displayed in Fig. 5. Notably, the highly asymmetrical animals were predominantly BoNT/A (BoNT/A vs. saline, $p = 0.037$ at

4 weeks and $p = 0.056$ at 12 weeks, Wilcoxon tests), but the side showing excessive labeling was often the uninjected side. The asymmetry sometimes appeared in the absence of condylar abnormalities (Fig. 6A,B), but in many cases, high counts were caused by heavy labeling of the fibrous tissue that filled defects in the condylar surface (Fig. 6C); as noted above, these defects could occur on either the injected or uninjected side but were more frequent in BoNT/A animals. These cases accounted for 6 of the 7 greatest asymmetries shown in Fig. 5. In contrast, the fibrous layer inclusions (Fig. 3C) observed in three BoNT/A animals (all uninjected side) lacked BrdU-labeled cells.

In summary (1) there was no age effect in condylar replication between the 4 week and 12 week groups (about 6.5 and 8.5 months old, respectively) and (2) in contrast to the original hypothesis, cartilage replication was not depressed on the underloaded BoNT/A injected side condyles. However, the elevated level of side-fluctuating asymmetry in the BoNT/A animals implies that one or both condyles had altered loading or outright damage.

DISCUSSION

Loading of the BoNT/A-injected side condyle was reduced

BoNT/A may affect the skeleton in several ways. Conceivably, the drug could have direct toxicity for skeletal cells, or the general inhibition of neurotransmitter release could affect other local signaling in the bone and cartilage. However, it is also possible that the effects reported here are simply consequences of modifying condylar loading. Evidence that the BoNT/A-side condyle was underloaded comes from a previous study in which we documented that EMG activity, masseter-induced bite force, and condylar bone strain were dramatically reduced on the BoNT/A side 3–4 weeks after masseter injection but mostly recovered by 11–12 weeks post-injection.¹⁵ The uninjected-side condyle was within normal limits for all parameters at all times, as was the saline-injected-side condyle. Loading of the injected-side condyle was reduced rather than removed, and the rabbits chewed normally,²⁶ presumably because the other jaw closing muscles (uninjected masseter plus medial pterygoids and temporales) remained active. The microCT measurements verified overall bone loss was restricted to the BoNT/A-side condyle, but BoNT/A group animals showed extreme variability at the 12-week timepoint, with some injected-side condyles showing minimal recovery and others showing more bone than the opposite, uninjected-side condyle.¹⁵ The current study provides information on the specific locations of bone loss and on the reaction of the condylar cartilage and also gives insight into why the variability in condylar bone measurements was so great at the 12-week endpoint.

Affected condyles lost cortical as well as trabecular bone

The high variability in bony content of the BoNT/A condyles seen in microCT scans¹⁵ was not seen in the specific regions examined for the present study (Table 2). In retrospect, this is because we did not previously identify the bony defect that was common on both sides of BoNT/A animals, especially at the 12-week endpoint. The loss of mineralized tissue in this area would have a considerable effect on microCT measurements, and because the defect often occurred on the uninjected side, this would account for the previous occasional anomalous findings of greater bone content on the injected side (see *Degenerative changes*

were seen in all samples but were increased in frequency in BoNT/A-treated animals for further discussion of this issue). However, the defect would not have affected the subchondral region examined in the present study because that region was defined by the osseous border itself.

Loss of bone is a consistent finding in response to BoNT/A-induced underloading^{12,15,27,28} and is usually thought to affect trabecular bone, with its greater marrow surface area, more severely than cortical bone. For example, BoNT/A paralysis of the murine hind limb led to a 44%–54% reduction in trabecular bone volume and 12%–20% reduction in cortical shell bone volume.²⁸ Although these microCT volume measurements are not directly comparable to the percent bone area and linear cortical thickness that were measured in the present histological study, a rough comparison can be made by converting all values to linear measurements (*i.e.*, cube root of volumes, square root of areas). By this measure, the rabbit condyle lost a similar amount of trabecular bone as the mouse hindlimb (4–6% in subchondral and midcondylar areas, respectively, vs. 4% in the mouse hindlimb) and showed much greater loss of cortical bone (15% vs. 2–3%). This greater cortical loss in the mandible than in the limb may be typical. After BoNT/A injection of the rat masseter, seven different sites on the mandible (not including the condyle) lost an average of 15% cortical bone thickness (calculated from reference 12). The strong osteopenic effect of muscle paralysis on the mandibular cortex likely relates to the fact that the mandible receives loading only through muscle contraction and not through weight bearing.

Trabecular bone loss was greater in the midcondylar than in the subchondral region

In contrasting the two trabecular areas of the condyle, we were testing the hypothesis that the subchondral region would undergo more loss than the midcondylar region. Our reasoning was that the subchondral region is located directly under the loadbearing cartilage, whereas the midcondylar region is relatively distant from it. This idea was clearly wrong. The midcondylar region showed more reduction and less recovery than the subchondral region. The most likely explanation for this regional difference is the less dense trabecular network in the midcondylar region. The midcondylar region initially had much sparser trabeculae, 40% bone area, than the finely trabeculated subchondral region, 60% bone area (Fig. 1, Table 2). The greater number of connections in the subchondral region implies that even if trabeculae lost continuity, they would still bear load through other connections, and could regain size when loading was again increased to normal levels. The sparser midcondylar region, on the other hand, would not have sufficient interconnections to transmit load from other trabeculae and thus trabeculae would be lost permanently if disconnected, as suggested by others.²⁹

Is it bone loss or failure to grow?

The mechanism of loss, at least in mouse tibia, is a rapid marrow osteoclastogenesis which occurs within 5 days.¹³ However, despite obvious osteopenia, we saw very few indications of resorption in any condyle. It is likely that 4 weeks post-injection was too late to observe active bone resorption. In addition, we cannot eliminate the possibility that condylar growth was still occurring in these young adult rabbits and some of the “loss” was instead a failure to grow. The continued cartilage proliferation and endochondral ossification, and the fine

trabecular structure observed in the subchondral region, imply that condylar growth might not have been complete in our sample, and others have noted histological changes in the rabbit condyle until even older ages.³⁰ Nevertheless, because 12-week endpoint condyles were similar in size to 4-week endpoint condyles,¹⁵ reduction in growth is an unlikely explanation for the loss of bone on the BoNT/A-injected side.

BoNT/A treatment did not affect replication of condylar chondrocytes

Because dietary underloading thins the condylar cartilage in adult as well as juvenile rats,¹⁹ and thinning was also seen in the single study (also on rats) using masseteric injections of BoNT/A,²⁴ we expected to see diminished condylar cartilage thickness in association with reduced cell divisions. Surprisingly, BoNT/A had no measurable effect on either parameter (Tables 2–4). Despite the dramatic bony changes, the condylar cartilage of the injected side was always similar to that of the uninjected side and that of the saline animals. This may reflect the fact that condylar position and movement were within normal limits during feeding, unlike the rat studies, which eliminated protrusion by trimming incisors and eliminated mastication by softening the diet. Since the condyle was definitely underloaded, as indicated by the loss of bone, this study supports the notion that underloading *per se* is not necessarily injurious to condylar cartilage.

Equally surprisingly, increasing animal age (about 6.5 months for the 4-week endpoint rabbits and 8.5 months for the 12-week rabbits) was associated with cartilage thickening. This result differs from the growth stasis reported at these ages elsewhere,³⁰ but that study measured thickness in the anterior condyle rather than the apex. The thicker condylar cartilage in the older animals is probably not associated with condylar growth, for reasons given above and the fact that there was no indication of increasing replication rate over time. Rather, the increased cartilage thickness was presumably due to matrix accumulation and may resemble the increase in the articular layer sometimes reported for human condyles.³¹

Degenerative changes were seen in all samples but were increased in frequency in BoNT/A-treated animals

Although proliferation rate in the condylar cartilage was not altered overall by BoNT/A treatment, there was a startling treatment effect in terms of the much higher incidence of degenerative morphology in the condyles of BoNT/A group animals (Fig. 3), specifically a large divot in the bony surface of the central/medial condyle. A relatively smooth articular surface was maintained over the gap by what appeared to be an extension of the fibrous layer of condylar cartilage. The appearance of this defect resembles “regressive remodeling” of the adult human mandibular condyle described as an early stage of TMJ osteoarthritis.³² The infilling tissue was usually mitotically very active, producing remarkable asymmetries in proliferation of right and left sides, suggesting a trauma-repair process. Other explanations seem less plausible. Because the defect occurred frequently on the uninjected side and was seen in two uninjected controls it was not an adaptive response to BoNT/A or to injection of the masseter. Nor was the defect a manifestation of immaturity despite its appearance in the relatively young control animals, because it was more frequent in the oldest (12-week) rabbits (Table 1). Speculatively, the initial damage might have been minor fracture of subchondral trabeculae, occurring on the BoNT/A side as a result of lack of bony

support, on the uninjected side as a result of abnormal loading, and in control animals as an sporadic traumatic event. It remains to be explained why the injected sides failed to show a statistically significant difference from saline/control rabbits ($p = 0.11$), unlike the uninjected sides and the combined results. Table 1 shows that the sides were equally affected at 4 weeks after injection (5/13 in both cases), but not at 12 weeks, when fewer injected sides (2/11) but more uninjected sides (7/11) showed the defect. It is, of course, possible that this observation was a statistical artifact; the difference is not significant ($p = 0.08$). If, however, the finding is real, it suggests that whereas the injected side may stabilize after the initial reactivation of the masseter, loading of the uninjected side may be exacerbated by the asymmetrical muscle forces which continue at least through 7 weeks.¹⁵

Potential clinical relevance

Unilateral and bilateral BoNT/A treatment of the masseter is commonly performed in the clinic, not only for muscle disorders, but also for pain relief and for cosmetic shaping of the face. Females, including young adults, make up the largest portion of such patients. If the present findings in young adult female rabbits can be extrapolated to humans, they suggest that even a single use of this paralytic drug may result in long-term bone loss in the mandibular condyle, potentially leading to future problems should the patient later develop osteoporosis or a TMJ disorder requiring surgery. Clinicians should consider mentioning this possibility at the time of obtaining consent for the procedure.

Conclusions

Inactivation of one masseter muscle from a single BoNT/A treatment in adult rabbits caused severe loss of cortical as well as trabecular bone in the mandibular condyle. The more sparsely trabeculated midcondylar region underwent greater loss than the densely trabecular subchondral zone. Although bone loss occurred only on the injected-side condyles, both sides of BoNT/A treated animals showed a high incidence of bony defects filled with a mitotically active fibrocartilaginous tissue. Other than this presumed trauma response and a thickening with age, the condylar cartilage remained of normal dimensions and replication rate and was not injured by BoNT/A-induced underloading. Importantly, neither the bone loss nor the frequency of the bone defect was significantly improved 12 weeks after the one-time injection. Condylar bone loss may be a risk factor for use of botulinum toxins in muscles of mastication.

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- A single dose of botulinum toxin to one masseter of rabbits caused extensive and long-lasting bone loss to the condyle of that side.
- A high frequency of condylar surface defects was seen on both treated and untreated sides.
- The condylar cartilage remained normal in thickness and proliferation rate.

Figure 1. Coronal section of a rabbit condyle showing measurements

A. Morphometrics. Condylar cartilage thickness (fibrous plus chondrocytic layers, CTh) was measured at the apex of the condyle. For purposes of replicating cell counts, the cartilage was divided into medial, central, and lateral thirds (defined by radial lines at 60° angles from a line passing through the condylar poles). Percent trabecular bone area was calculated from the subchondral (SC) and midcondylar (MC) regions (shaded). The subchondral region was defined by shifting the outline of the superior curvature of the condylar bone inferiorly by 750 μm ; the resulting subchondral area was always 750 μm in maximum height but varied in the other dimensions with individual condyle size and shape. The mid-condylar region was defined by a 750 $\mu\text{m} \times 750 \mu\text{m}$ area directly inferior to the subchondral region and in line with the apex. Cortical thickness was measured on medial (MTh) and lateral (LTh) sides of the condylar neck perpendicular to the long axis of the cortex and 1500 μm from the most inferior aspect of the midcondylar regions. **B.** A 50 $\times$ 50 grid, 5.3mm $\times$ 5.3mm, was superimposed on the image. Percent trabecular bone area was calculated as the number of intersections overlying bone (black dots) divided by the total number of intersections in each region.

Figure 2. Zones of the condylar cartilage

The central third of a condyle (BoNT/Ainjected side, 4-week endpoint) is shown in a BrdU-reacted section (methyl green counterstain). Labeled cells were rare in the fibrous zone (Fib Z) but common at the lower part of the proliferative zone (Pro Z) and its junction with the mature plus hypertrophic zones (M&H Z), as well as at the mineralizing bone front.

Figure 3. Condylar morphology

A. Typical appearance in coronal section (injected side of a saline 4-week endpoint rabbit, hematoxylin and eosin). Note the rounded shape with apex toward the medial (right) side. **B.** Articular flattening and a bony defect with fibrous infilling (arrow) in the injected side of a BoNT/A 12-week endpoint rabbit (hematoxylin and eosin). **C.** Inclusion in the fibrous layer of the articular cartilage (uninjected side of a BoNT/A 12-week endpoint rabbit, hematoxylin and eosin).

Figure 4. Injected vs. uninjected side comparisons of bone areal percentage in the subchondral and midcondylar regions (Y-axis on left, black) and cortical bone thickness in the condylar neck (Y- axis on right, red)

The figure plotted is the uninjected side value minus the injected-side value (mean and standard error). In the BoNT/A animals, at both endpoints, the values were all positive, more bone for the uninjected side whereas values for the saline animals were close to zero or negative.

Figure 5. Side asymmetry in BrdU-positive cell counts

Positive values indicate animals that had more positive cells on the injected side, whereas negative values indicate more positive cells on the uninjected side. Each symbol represents one rabbit. BoNT/A animals (shaded columns and symbols) were more asymmetrical than saline animals (open columns and symbols) ($p = 0.037$ for the 4-week endpoint and $p = 0.056$ for the 12-week endpoint).

Figure 6. Asymmetrical replication in the condylar cartilage of BoNT/A group rabbits

A and B. Injected and uninjected sides, respectively, of a 4-week endpoint animal. There are no obvious signs of pathology, but the uninjected side shows much a higher level of labeling (arrows). **C.** Labeling was typically high in the fibrous infill of condylar defects (injected side of a 4-week endpoint animal). BrdU reaction and methyl green counterstain.

Table 1

Subjective observations of condylar defects

Treatment/Time	Affected^a Rabbits / Total Rabbits	Injected Side Bony Defect / Total Condyles	Uninjected Side Bony Defect / Total Condyles	Sides Combined Bony Defect / Total Condyles
BoNT/A, 4 weeks	6/13 (46%)	5/13 (38%)	5/13 (38%)	10/26 (38%)
BoNT/A, 12 weeks	9/11 (64%)	2/11 (18%)	7/11 (64%)	9/22 (41%)
Combined	15/24 (63%)	7/24 (29%)	12/24 (50%)	19/48 (40%)
Control, 0 weeks	2/3 (67%)	NA	NA	3/6 (50%)
Saline, 4 weeks	1/7 (14%)	0/7 (0%)	1/7 (14%)	1/14 (1%)
Saline, 12 weeks	2/9 (22%)	1/9 (11%)	1/9 (11%)	2/18 (11%)
Combined	5/19 (26%)	1/16 (1%)	2/16 (1%)	6/38 (16%)
<i>BoNT/A vs Control+Saline^b</i>	<i>p = 0.03</i>	<i>p = 0.11</i>	<i>p = 0.02</i>	<i>p = 0.02</i>

^a Affected condyles differed from normal in having a bony defect filled with cartilage and connective tissue and/or an irregular contour.

^b Fisher's exact test.

Table 2

Condylar morphometry (mean [standard deviation])

Treatment/Time	Side				Side			
	n	Injected	Uninjected	p, paired t-test	n	Injected	Uninjected	p, paired t-test
Subchondral Trabecular Bone Areal Density (%)								
BoNT/A, 4 weeks	10	52.1 [3.9]	63.8 [5.7]	<0.01	10	26.0 [7.1]	45.7 [14.2]	<0.01
Saline, 4 weeks	7	64.4 [5.3]	61.7 [5.8]	0.44	7	41.2 [9.3]	43.4 [12.4]	0.59
p, 2 sample t-test		<0.001	0.58			0.01	0.62	
BoNT/A, 12 weeks	10	55.5 [8.1]	60.2 [9.9]	0.11	10	28.5 [10.3]	38.9 [15.1]	0.01
Saline, 12 weeks	8	61.7 [7.7]	59.3 [8.7]	0.44	8	35.6 [16.7]	34.9 [16.9]	0.85
p, 2 sample t-test		0.07	0.86			0.22	0.68	
Condylar Neck Cortical Thickness (medial plus lateral, µm)								
BoNT/A, 4 weeks	9	836.3 [132]	1010.9 [216]	0.06	10	234.1 [59]	262.0 [70]	0.37
Saline, 4 weeks	6	1208.8 [248]	1126.8 [132]	0.46	5	219.6 [46]	257.7 [30]	0.32
p, 2 sample t-test		0.03	0.20			0.69	0.75	
BoNT/A, 12 weeks	9	926.1 [80]	1099.2 [204]	0.01	7	340.8 [88]	315.4 [80]	0.49
Saline, 12 weeks	6	1010.3 [109]	977.6 [200]	0.78	7	331.7 [90]	340.9 [82]	0.87
p, 2 sample t-test		0.09	0.26			0.81	0.55	
Condylar Cartilage Thickness (all layers combined, µm)								

Adjusted ANCOVA for treatment (BoNT/A vs. saline) and time (4 weeks vs. 12 weeks post-injection) effects on the mandibular condylar bone and cartilage

Table 3

Outcome Variable	Mean Difference	95% Confidence Interval for Difference		p-value	Significance Direction
		Lower	Upper		
Trabecular areal density: subchondral (%)					
Treatment	-9.6	-14.0	-5.3	<0.001	BoNT/A < Saline
Time	-1.8	-6.2	2.6	0.418	--
Trabecular areal density: midcondylar (%)					
Treatment	-12.4	-18.9	-5.9	<0.001	BoNT/A < Saline
Time	-2.5	-9.1	4.1	0.451	--
Cortical thickness (µm)					
Treatment	-226.7	-353.4	-100.1	0.001	BoNT/A < Saline
Time	26.8	98.0	151.6	0.662	--
Cartilage thickness (µm)					
Treatment	14.4	-44.7	73.5	0.620	--
Time	-95.0	-159.0	-31.0	0.005	4wk < 12wk
Cell proliferation (number of BrdU+ cells/condylar cartilage)					
Treatment	-27.2	-71.2	16.8	0.218	--
Time	35.4	-7.6	78.5	0.104	--

Table 4

Counts of BrdU-Labeled Cells in Condylar Cartilage

Treatment/Time	Injection Side			Uninjected Side		
	n	Mean $\pm$ SD	Median (Min-Max)	n	Mean $\pm$ SD	Median (Min-Max)
BoNT/A, 4 weeks	13	50 $\pm$ 39	34 (12–136)	13	40 $\pm$ 47	18 (6–166)
Saline, 4 weeks	7	42 $\pm$ 20	40 (12–68)	10	44 $\pm$ 27	30 (16–94)
BoNT/A, 12 weeks	11	58 $\pm$ 60	36 (11–200)	11	100 $\pm$ 116	54 (4–419)
Saline/12 weeks	9	49 $\pm$ 69	33 (7–228)	9	44 $\pm$ 44	37 (7–143)