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Botulinum toxin in masticatory muscles: Short- and long-term effects on muscle, bone, and craniofacial function in adult rabbits

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Abstract

Paralysis of the masticatory muscles using botulinum toxin (BTX) is a common treatment for cosmetic reduction of the masseters as well as for conditions involving muscle spasm and pain. The effects of this treatment on mastication have not been evaluated, and claims that the treatment unloads the jaw joint and mandible have not been validated. If BTX treatment does decrease mandibular loading, osteopenia might ensue as an adverse result. Rabbits received a single dose of BTX or saline into one randomly chosen masseter muscle and were followed for 4 or 12 weeks. Masticatory muscle activity was assessed weekly, and incisor bite force elicited by stimulation of each masseter was measured periodically. At the endpoint, strain gages were installed on the neck of the mandibular condyle and on the molar area of the mandible for *in vivo* bone strain recording during mastication and muscle stimulation. After termination, muscles were weighed and mandibular segments were scanned with micro CT. BTX paralysis of one masseter did not alter chewing side or rate, in part because of compensation by the medial pterygoid muscle. Masseter-induced bite force was dramatically decreased. Analysis of bone strain data suggested that at 4 weeks, the mandibular condyle of the BTX-injected side was underloaded, as were both sides of the molar area. Bone quantity and quality were severely decreased specifically at these underloaded locations, especially the injection-side condylar head. At 12 weeks, most functional parameters were near their pre-injection levels, but the injected masseter still exhibited atrophy and percent bone area was still low in the condylar head. In conclusion, although the performance of mastication was only minimally harmed by BTX paralysis of the masseter, the resulting underloading was sufficient to cause notable and persistent bone loss, particularly at the temporomandibular joint.

Keywords

Botulinum neurotoxin; masseter; mastication; mandibular bone strain; bone microarchitecture; temporomandibular joint

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1. Introduction

For muscles of the limbs and trunk, clinical treatment with botulinum toxins (BTX) is reserved for serious conditions of muscle spasm and contracture [1-3], but in the face these paralytic toxins are freely used for asymptomatic as well as hypertonic muscles. In addition to injecting facial muscles to relax wrinkled skin and relieve blepharospasm, BTX is used to inactivate the major muscles of mastication, particularly the large masseter muscles. One common reason for injecting BTX into asymptomatic masseters is cosmetic, to change a square-angled face into a more oval one by causing the muscles to atrophy; patients receiving this treatment are predominantly young and female and typically receive repeat injections [4-6]. The masseters are also paralyzed with BTX when it is considered desirable to unload the jaws [7-9] and in the hope of alleviating facial pain involving the temporomandibular joint (TMJ) [10], although load reduction has not been proven and relief of pain is marginal [10-12].

When used experimentally on limb muscles, BTX produces dramatic bone loss in the tibia and distal femur, which lingers long after ambulation normalizes [13-16]. Such osteoporotic changes presumably result from disuse unloading, comparable to space flight or bed rest. Concern about bone loss has not been a feature of clinical articles dealing with BTX in craniofacial muscles, perhaps because the skull does not bear body weight and under some conditions appears to be insensitive to mechanical loading [17]. However, the skull is certainly sensitive to muscular loading, as shown by rapid osteopenia when muscle loading is reduced by a soft diet [18]. The mandible in particular is dominated by the attachments of the large, strong muscles of mastication. Nevertheless, clinical studies with BTX in masticatory muscles have ignored the issue of bone quality, and animal studies have addressed it mainly in the context of skull growth [19-23].

There is ongoing discussion of whether the osteoporotic effect of BTX-induced paralysis is solely through loss of muscle loading or partly due to decrease in weight bearing [13-16, 24, 25]. Most authors suspect that muscle paralysis alone suffices, but separating hind limb muscle disability from weight bearing is difficult, and the absence of electromyography (EMG), muscle force, and bone strain data from most studies has left many questions unanswered. The fact that the mandible is non-weight bearing provides a new approach to isolating the primary vs. secondary effects of BTX-induced paralysis on bone. The mandible functions as a lever system in which adductor muscle force is countered by a temporomandibular joint reaction force as well as an occlusal bite force. Thus both the mandibular condyle and the tooth-bearing body of the mandible receive their loading strictly from muscle activity. Furthermore, EMG, bite force, and *in vivo* bone strain are relatively easy to measure with the muscles of mastication and the mandible, so that full data on function can be obtained.

Despite a plethora of clinical reports, there is strikingly little information in the literature about how BTX paralysis of jaw muscles affects mastication nor about the consequences for the mandible. A few authors have looked at maximum bite force and its associated EMG. Surprisingly, these studies have indicated that EMG and bite force recover much faster than muscle volume, sometimes eventually reaching levels higher than the initial level [4, 26-27]. Difficulty in chewing is anecdotally reported to last only 1-2 months and primarily to involve hard or tough foods [4]. These findings suggest that other jaw muscles have compensated for the loss of the masseter, although the limited data available do not show evidence of this. Specifically, the temporalis and medial pterygoid do not enlarge after masseter injection [27, 28], and injection of the temporalis as well as the masseter does not hinder the recovery of bite force [29, 30]. Animal studies to date have not included any EMG or measurements of mastication or force, nor do they address the issue of

compensatory activity in other muscles. Thus the potential translational and scientific value of a masticatory muscle animal model for BTX has not yet been realized.

The present study was undertaken to answer the following questions. (1) What are the functional consequences of BTX-induced paralysis of the masseter, particularly in regard to mastication? In particular, do other muscles compensate for the loss of one masseter? (2) What is the relationship between EMG activity, muscle force and muscle volume? (3) Does this treatment unload the temporomandibular joint (TMJ) and/or the tooth-bearing region? (4) Do osteoporotic changes occur at either the mandibular condyle or the alveolar region of the mandibular body? (5) Do loss and recovery of muscle and bone function and structure occur in parallel? We used rabbits to take advantage of their well-known masticatory and EMG patterns [31-35], their previous use in BTX studies [19], and reasonable similarity to humans in masseter morphology and jaw movement [31, 34, 35]. We expected to see that functionally similar muscles (e.g. medial pterygoid) would compensate for paralysis of the masseter muscle, that EMG activity, force and bone strain would all be reduced 4 weeks after paralysis but show recovery at 12 weeks, and that bone quantity and quality of the mandible would be degraded and would recover relatively poorly.

2. Methods

2.1 Animal model and in vivo measurements

A summary of the study design is provided in Fig. 1. All procedures were approved by the University of Washington Animal Care and Use Committee. Female New Zealand white rabbits (n= 41, 5 months old, 3.8 - 5.1 kg) were obtained in cohorts of 8-9 animals. They were maintained on a 12:12 light/dark schedule and received 225g of pellets (Albers® Rabbit 16% Animal Feed) per day. Weight was monitored 5 days/week. Anesthesia (isoflurane by mask) was used during injection of the masseter muscle, electrical stimulation of the masseter to measure bite force, insertion of electrodes for fine-wire electromyography (EMG), and for procedures on the terminal day.

Rabbits were acclimated to the laboratory environment for about 2 weeks. Baseline functional assessments were made of chewing behavior and masseter-induced bite force. Surface and fine-wire EMG were carried out on separate days, and both were accompanied by synchronized video (QuickCam, Logitech, Fremont CA), which was used to determine chewing side. Surface EMG utilized pairs of snap electrodes (Vermed, Bellows Falls VT) affixed to the shaved skin overlying each superficial masseter muscle and a ground surface electrode attached to the shaved forehead. No anesthesia was required. Animals were fed their usual pelleted diet. EMG signals were recorded to computer at an acquisition rate of 1kHz using the MP150 data acquisition interface and the software AcqKnowledge (BIOPAC Systems, Goleta CA). For fine-wire EMG, anesthesia was used and baseline tetanic bite force produced by the masseter muscles was measured at the same session. Sterile bipolar wire electrodes (0.05 mm nickel-chromium alloy with 1 mm exposed tips) were inserted bilaterally via 27- gauge needles into three locations in the superficial masseter (anterior, middle, and posterior), the deep masseter, and the medial pterygoid (Fig. 2A). The anterior and posterior superficial masseter electrodes were connected to a stimulator (Grass S48 and SIU5, Astro-Med, West Warwick RI or Biopac STM100C/STMIOC) and used to tetanize each masseter (5 ms pulses, 55 pps, 550 msec trains, voltage gradually increased until supra-maximal tetany was achieved, usually at 20-30 V) while bite force at the incisors was measured. The bite force transducer, constructed from two aluminum beams and 4 single element strain gages in a full bridge configuration [36], was placed between the incisor teeth. The transducer was calibrated using a materials testing machine (Syntech 2/S, MTS, Eden Prairie MN). Following these measurements, electrodes were connected to recording

equipment, the gas anesthesia was removed, and the animals were allowed to awaken and given food.

EMG recordings were analyzed in AcqKnowledge using the same procedures for surface and wire recordings. Noise was removed with a 60 Hz band stop, followed by filtering (bandpass 60-500 Hz, Hanning window with $Q=32$), rectification and 3-point smoothing. The duration of the burst was defined as deflection of the baseline by 10% of the peak amplitude for the session. The mean amplitudes of 15-20 consecutive masticatory bursts were calculated for each muscle or muscle part and each side of chewing. The fine-wire EMG recordings were also analyzed for the timing of onset and offset of each burst, taking the onset of the posterior location of the uninjected masseter as zero.

In the following week (designated week 0) animals received a unilateral masseter injection of botulinum toxin type A (Botox[®], Allergan, abbreviated BTX below) or 0.9% saline. The treatment and the side were determined by coin flip, as was assignment to endpoint group (4 weeks or 12 weeks). Researchers were blinded to these designations. BTX (100 units) was reconstituted with normal saline according to the package insert. Each injected masseter received 10 units (0.25ml) divided equally into anterior, middle, and posterior locations of the superficial masseter (Fig. 2A). The saline animals received the same volume injected in the same locations. These locations were chosen to approximate the location of the motor end plates of the superficial masseter [35]. The dosage of 10 units of BTX is the same as in previous rabbit studies [19] and, relative to masseter weight (1-2 units/g muscle), corresponds to the lower end of the typical dose for humans (1-5 units/g muscle, assuming 25-50 units [7] injected into a masseter weighing 7-21 g [37]). The injection sites were briefly massaged to encourage dispersion of the injected fluid. The unused reconstituted BTX was drawn up in syringes of the appropriate dose and frozen immediately for use in the following round of animals (approximately 3 months later). This use of frozen toxin is counter to the manufacturer's directions, but clinical studies have suggested that potency is retained for up to 6 months [38], and our comparisons of masseter-induced bite force and other parameters revealed no significant differences between muscles injected with fresh vs. frozen reconstituted BTX.

In subsequent weeks (except for week 4), wire and surface EMG with video were recorded as for the baseline measurements. Masseter-induced bite force was measured at week 3 (all animals) and weeks 7 and 11 (for 12-week endpoint animals). On the terminal day (both endpoints), most animals underwent a surgical procedure to apply strain gages to the neck of the condyle and the alveolar process on both sides (Fig. 2B). The neck of the condyle was exposed by gently retracting the posterior fibers of the deep masseter anteriorly. The alveolar site was subcutaneous and was approached through an incision on the lower border of the mandible. Each exposed surface was cleaned, neutralized and dried, and then a rosette strain gage (SK-06-030WR-120, Vishay Micro-Measurements, Raleigh NC) was applied to the bone with cyanoacrylate glue. Prior to suturing the flap, the orientation of each gage was measured relative to the occlusal plane. The surgery took approximately two hours. The strain gages were connected to conditioner/amplifiers (2100 system, Vishay) and the signals led to the Biopac recording apparatus. Wire EMG electrodes were inserted as usual and analgesics were administered. Initially buprenorphine (0.01-0.02 mg/kg IM) was used, but this proved soporific, and later ketorolac (1 mg/kg IM) was substituted. The animals were then allowed to awaken and were fed. EMG, video and strain were recorded during mastication similar to a fine-wire EMG session. After 10-20 minutes of feeding, the rabbit was re-anesthetized and strain recordings were made during stimulation of the masseter muscles. The bite force transducer was not used during these stimulations, and the teeth were in occlusion. EMG data were analyzed as above. Strain data were processed by subtracting baseline voltage from peak masticatory or stimulated voltage from each channel,

and then values from the 3 elements of each rosette were used to calculate the magnitude and orientation of the principal maximum (tensile) and minimum (compressive) strains (Rosette-Plus, Vishay); peak shear strain was calculated by subtracting minimum from maximum principal strain [39].

2.2 Muscle and bone specimens

After recording, the animals were euthanized by cardiac perfusion with 0.9% normal saline followed by 4% paraformaldehyde. Heads were stored in 4% paraformaldehyde. After verifying strain gauge position, the muscles of mastication were removed, blotted dry and weighed. The mandibular condylar processes were removed using a Dremel tool to cut through the condylar neck 1cm below the head and parallel to the occlusal plane. Alveolar specimens were removed by vertical cuts through the mandibular body at the anterior and posterior borders of the tooth row and thus included the entire molar region (Fig. 2B). A Viva CT40 (Scanco, Brüttisellen, Switzerland) was used to obtain 21mm voxel resolution images of the condylar process and molar region. The condylar process was scanned in the dorso-ventral direction, beginning at the articular surface and moving ventrally to a distance of 8 mm; the anteroposterior dimension was set at 11 mm. For molar region specimens the midpoint of the tooth row was determined and 200 coronal slices were analyzed, 100 anterior and 100 posterior to the midpoint. Tooth roots were defined by manually drawn contours every 10 slices (with Scanco interpolation software used between these slices) and were excluded from analysis. Signal noise was reduced with a Gauss filter (Scanco sigma and support parameters: 1.2 and 2.0, respectively). For both condylar and alveolar specimens, tissue volume (TV, mm³), bone volume (BV, mm³), and bone volume fraction (BV/TV, %) were determined using a fixed threshold of 400. This threshold was established in preliminary studies as optimal to capture bone trabecular and cortical bone contained within the samples.

To examine the subarticular area of the condylar process (“condylar head”) in isolation, the condylar scans were digitally resliced in the coronal plane (DATAVIEWER, SkyScan, Kontich, Belgium). The condylar head has clear medial and lateral landmarks, the poles of the condyle, but lacks definitive anterior and posterior landmarks. Therefore, the condylar head was analyzed as an area, not a volume. Three slices from the coronal center (identified by the presence of medial and lateral poles) were chosen. In each slice the condylar head was defined by the superior border of the specimen and a horizontal line drawn between the medial and lateral poles (see outlined area in Fig. 8A). The condylar head, thus defined, was entirely trabecular in all cases. These three slices were analyzed two-dimensionally (CT-Analyser, SkyScan) and mean values were calculated for each side/individual. Measurements included tissue area, bone area, percent bone area, and structural indices of trabecular architecture [40]. Each trabecular parameter (trabecular number, thickness, and separation) was calculated separately for a model assuming the trabeculae form parallel plates and a model assuming cylindrical rods; the two models showed similar trends, but with different values. Because trabeculae are a mix of irregular plates and rods, neither model is likely to yield true values. The software calculated all parameters, including the derived trabecular indices, using the standard definitions and formulae summarized by Parfitt et al. [40].

2.3 Statistical analysis

The data were explored using statistical tests performed in Excel and SPSS. Proportional data (e.g. BV/TV) were arcsine transformed prior to statistical analysis in order to equalize the variance [41]. Paired t-tests were used to compare injected and non-injected sides within BTX and saline groups, and two-sample t-tests were used to compare the BTX and saline animals. Kruskal-Wallis ANOVAs were used to search for changes in timing of EMG

activity. Because of the multiplicity of tests performed, significance levels are advisory only, and we drew conclusions only when several lines of evidence led to the same finding.

3. Results

3.1 Body weight and mastication

BTX-induced paralysis of one masseter had no effect on body weight; week 3 weights were 4.04 ± 0.27 in the toxin group and 3.96 ± 0.36 kg in the saline group, and at week 11 they were 3.86 ± 0.35 kg and 3.85 ± 0.38 kg, respectively. Masticatory rate was unchanged (3.31 ± 0.33 Hz for the toxin group at week 3, 3.41 ± 0.29 Hz for the saline group), and rabbits continued to chew on both sides of the jaw. If anything, animals tended to chew more on the paralyzed side at the height of toxin effect ($58 \pm 24\%$ of cycles at week 3, $p = 0.5$, in contrast to 46-54% for other weeks and saline-injected muscles).

Because mastication is asymmetrical in rabbits, working-side (the side with the food bolus) and balancing-side (the opposite, empty side) cycles were treated separately. Normal values for surface EMG are represented by the uninjected masseter of the saline controls, for which the overall average (all timepoints) was 66.8 ± 13.2 μ V burst amplitude when on the working side and 57.9 ± 5.9 μ V when on the balancing side (calculated from Supplement A). The uninjected side of the BTX animals did not differ from these normal values (60.6 ± 9.3 μ V working, 52.6 ± 12.1 μ V balancing). The saline-injected masseters were somewhat lower than the uninjected muscles of the same animals when on the working-side (57.2 ± 8.2 μ V, $p = 0.01$ in paired t-test) but slightly higher when on the balancing side (61.3 ± 8.4 μ V, $p = 0.13$). Regardless of chewing side, the BTX-injected masseters had significantly lower average activity than the saline-injected masseters and the contralateral uninjected muscles at weeks 1 and 2 after injection, and usually during week 0 (a few days after injection) and week 3 as well. The lowest value, at 2 weeks, was 17.1 ± 10.4 μ V ($p = 0.0005$ compared to the contralateral muscle, 53.0 ± 32.6 μ V, Supplement A). When on the working side, activity remained low in comparison to the uninjected side throughout the 11-week study (Fig. 3). For example, at 10 weeks the BTX-injected masseter averaged 36.0 ± 16.9 μ V compared to the uninjected side at 53.6 ± 14.0 μ V (Supplement A, $p = 0.04$).

Fine-wire EMG recordings (Fig. 4) verified the precipitous drop in the amplitude of recordings from the BTX-injected superficial masseter after injection (e.g., for the anterior location, from 136 ± 90 μ V at baseline to 6 ± 4 μ V at weeks 1-2, Supplement B), but interestingly, these were not uniform. The anterior and middle locations of the superficial masseter were greatly affected at weeks 1-2, whereas the posterior location was not reduced until weeks 5-6. Another interesting trend was for reduction in EMG of non-targeted muscles (deep masseter and medial pterygoid) on the injected side at weeks 1-2, normalizing by weeks 5-6. At weeks 9-11, the anterior location of superficial masseter was fully recovered, but the middle location still tended to be low. In contrast to the BTX-injected side, the muscles of the uninjected side showed no clear trends over time, nor did either the injected or uninjected side of the saline controls (Supplement B).

The temporal pattern of muscle contraction showed a single but notable change following BTX injection into the masseter. At baseline, on the uninjected side and on both sides of the saline controls, the onset times of all the recorded muscles were similar to those of the reference muscle (the uninjected posterior superficial masseter). However, at weeks 1-2 after BTX injection into the masseter, the medial pterygoid muscle on the side of the injected masseter had an earlier onset regardless of the side of chewing (Fig. 5; $p = 0.007$ in a Kruskal-Wallis comparison of time periods, the only such comparison to show $p < 0.05$).

3.2 Incisor bite force production by the masseter

Measurements of incisor bite force produced by direct stimulation of the masseter in anesthetized animals demonstrated that the loss of force after BTX was profound and longer lasting than the loss of EMG activity in the same muscles. Figure 6 presents bite force produced by the injected masseter as a percentage of that produced by uninjected side (data in Supplement C). At no time did saline injection affect masseter-induced bite force, nor did the uninjected masseter of BTX animals ever differ from baseline levels of about 1.6 kg. However, the BTX-injected masseter only produced about 0.2 kg at week 3, a reduction of over 85% ($p < 0.0001$), and at week 7 the average masseter-induced bite force was improved only to 0.5 kg ($p = 0.002$). By week 11, the difference was no longer present.

3.3 Muscle weights

At the 4-week endpoint, the masseter muscle injected with BTX was on average 18% lighter than the contralateral muscle ($p < 0.0001$), and atrophy averaging 7% was still observable at the 12-week endpoint (Table 1). The non-injected side was similar to saline controls. The medial pterygoid muscle on the BTX-injected side also weighed 10% less than on the uninjected side ($p < 0.001$), but comparison with the saline controls indicates that this was due to hypertrophy of 25% ($p = 0.02$) on the non-injected side rather than atrophy of the injected side medial pterygoid, and statistical significance of this difference was gone at 12 weeks. Temporalis and lateral pterygoid are very small muscles in rabbits; there were no side or group differences at 4 weeks, but a tendency for the injected side temporalis of the BTX animals to be smaller than the non-injected side was seen at 12 weeks (Table 1).

3.4 Bone strain during mastication and muscle stimulation

Sample size was small for strain recordings, mostly because of instrumentation problems. Mastication data are presented in Table 2. Saline animals showed no effects of endpoint or injection, so data from both sides of 4-week and 12-week animals were combined, creating adequate control sample sizes of 9-10. These control values show that the molar region on the working side of the jaw (the side with the food bolus) tended to have higher strains than the balancing side ($408\mu\epsilon$ vs. $306\mu\epsilon$), but both sides had similar orientation of principal strains, with the compressive strain directed from anterosuperior to posteroinferior (Fig. 7). However, the side of chewing did not affect strain on the condylar neck, which showed similar shear strain magnitudes on the working and balancing sides ($385\mu\epsilon$ and $343\mu\epsilon$) as well as identical orientations of strain with compression directed vertically (89° and 96° , Fig. 7).

Groups and sides could not be combined for BTX animals, resulting in poor sample sizes of only 1-6. For this reason, statistical tests are not presented in Table 2. Nevertheless, compared to control values, the BTX animals showed the following trends: (1) in the molar region, both sides of the jaw had low values of shear strain at both the 4-week and 12-week endpoints ($187\text{--}318\mu\epsilon$ vs. the control average of $408\mu\epsilon$ for the working side; $141\text{--}215\mu\epsilon$ vs. the control average of $306\mu\epsilon$ for the balancing side, Table 2); (2) both sides of the molar region showed an abnormal anteroinferior to posterosuperior orientation of balancing-side compressive strain at the 4-week, but not the 12-week endpoint; (3) strain levels at the condylar neck tended to be low, but only on the injected side ($216\text{--}348\mu\epsilon$ for BTX-injected averages vs. $343\text{--}610\mu\epsilon$ for uninjected side and control averages); (4) compressive strain on the injected side condylar neck was abnormally oriented from anteroinferior to posterosuperior at the 4-week endpoint.

The strains produced by electrical stimulation of one masseter muscle at a time are shown in Table 3. The sample size problem for the BTX animals persisted, although these data adequately show that masseter-induced strain on the mandible fell 4 weeks after BTX

injection and was mostly recovered at 12 weeks. Interestingly, shear strains were highly variable at 12 weeks and sometimes very high, and orientations were also very irregular compared to control values.

Stimulation of control muscles showed that the masseter of one side produces considerable strain on both sides of the jaw, but these strains are not identical. For the molar region, there was little difference between sides in shear strain magnitude ($241 \mu\epsilon$ vs. $173 \mu\epsilon$, $p = 0.15$), but a distinct change in orientation. Notably, the strain orientations produced by contraction of the opposite masseter (145° , Table 3) were similar to the abnormal balancing side of the 4-week BTX animals during mastication (137° - 148°). In contrast to the molar regions, the condylar necks showed differential shear strain ($367 \mu\epsilon$ vs. $207 \mu\epsilon$, $p = 0.002$) as well as altered orientation when control masseters were tetanized.

3.5 Micro CT analysis of the mandibular body and condylar process

The alveolar section of the mandibular body maintained constant tissue volume after BTX injection, but at the 4-week end point, percent bone volume was decreased from saline controls (60%) on both the injected (54%) and uninjected (56%) sides ($p \leq 0.01$, Table 4), with a trend for the injected side to show a greater loss than the uninjected side ($p = 0.04$). Although these trends were still apparent at 12 weeks, the differences were no longer statistically significant.

Unlike the alveolar bone, the mandibular condyle only showed changes on the BTX-injected side. The uninjected side was indistinguishable from the saline controls in all parameters (Table 4). However, the differences between the sides were dramatic and long-lasting. Bone volume and percent bone volume (for whole specimens) and bone area and percent bone area (for coronal sections of the condylar head, Fig. 8) were strongly reduced on the side of BTX injection compared to the uninjected side at 4 weeks ($p \leq 0.0001$, Table 4), and the differences remained observable at 12 weeks. On average, the percent bone area of the injected-side condylar head was 42% less than the opposite side at 4 weeks and 15% less at 12 weeks (calculated from Table 4). The BTX -injected side condyles were also reduced in comparison to saline controls at 4 weeks with the similar trends persisting at 12 weeks. Interestingly, although the non-injected side was never significantly different from saline controls, it tended to be larger in all parameters, notably including overall size as measured by tissue volume/area. This trend accentuated the difference between the sides in the BTX animals and caused significant side differences in tissue volume/area at 4 weeks.

The parallel plate model findings for trabecular architecture in the condylar head are presented in Table 5. The rod model showed the same trends, but diameter measurements were approximately twice as large as the plate thickness values, rod separation values were lower and more variable than plate separation values, and rod trabecular numbers were lower and less variable than the plate trabecular numbers. Most statistical tests gave the same results with both models. As with measures of bone area and percent bone area, the non-injected side of the BTX rabbits was always within the range of saline controls, but the injected side showed dramatic and persistent reductions in trabecular bone. The BTX -injected side condyle had thinner and fewer trabeculae relative to both the opposite side and to saline-injected controls, and at 4 weeks trabecular separation was almost triple the normal value ($58 \mu\text{m}$ vs. $20 \mu\text{m}$). Although notably improved at 12 weeks ($p \leq 0.03$ in comparisons of 4 week vs. 12 week BTX-injected sides, except for trabecular number under the rod model, $p = 0.12$), almost all of these differences remained statistically significant (Table 5).

4. Discussion

4.1 Masseter paralysis and mastication. Do other muscles compensate?

Because all rabbits received the same daily ration, no difference in weight would be expected even if BTX harmed food intake, which is unlikely based on previous studies [21, 23]. Somewhat more surprising is that chewing was so little altered by a treatment that dramatically diminished the EMG and force of one of the largest muscles of mastication. The rabbits seemed unaware of their functional deficit, continuing to chew on both sides at an unaltered rate, with what appeared to be normal jaw movement.

To some extent, the normality of mastication in the BTX injected animals may simply indicate that the pelleted rabbit food was easily pulverized. In rabbits, in contrast to humans, chewing side and movement do not depend strongly on any particular muscular contraction pattern, because the occlusal surfaces of the teeth are strongly inclined; provided the teeth are brought into occlusion, any closing muscle(s) will move the mandible toward the midline. In addition, the medial pterygoid muscle compensated at least in part for the BTX-paralyzed masseter. The early onset of activity in this muscle on the BTX side probably provided the initial occlusal positioning, while increased force was provided by the hypertrophied contralateral muscle. In this regard, it is peculiar that the injection-side medial pterygoid was not equally hypertrophied (Table 1). One possible explanation is that this muscle and the injection-side temporalis were affected by BTX leakage from the target muscle [42], preventing greater compensation.

In humans, mastication is guided less by occlusal morphology and more by patterns of muscle activation, so humans should have greater disruption of chewing after BTX injection into the masseter, especially because compensatory hypertrophy of other muscles apparently does not occur [27, 28]. Cosmetically treated patients do report “uneven bumpy muscular movements” as well as diminished power [4]. Thus, although masticatory function may improve in patients with jaw muscle spasm [29, 43], it worsens temporarily in normal patients.

4.2 Masseter activity, mass, and force

Muscle mass is not an ideal estimate of force. In addition to physiological cross section, muscle mass is influenced by factors including the content of non-contractile fibrotic or fatty tissue, which accumulates after BTX injection [4, 44]. In the present study we also stimulated the masseter muscle and recorded incisor bite force, providing a more direct measurement of functional capacity. However, functional capacity does not necessarily reflect actual usage of the muscles, so we tracked muscle activity during mastication with EMG. This combination of techniques has not been attempted previously and affords a unique opportunity to examine many facets of muscle function and assess their influence on bone.

Importantly, each of these measures of muscle function indicated that only BTX injections had an effect; saline-injected masseters were similar to their non-injected control sides, and the non-injected masseters of the BTX animals were also within these normal parameters. Thus there is no reason to believe that the BTX had any systemic consequences, either negative (from the toxin) or positive (such as hypertrophy). Use of reconstituted BTX that had been frozen for 3 months in some rabbits did not affect the results in specific comparisons of bite force and muscle weight. Thus, as reported by others [38], efficacy is maintained at least for a few months. It remains possible that the toxin had lost some activity, but that our procedures were not sufficiently sensitive to detect differences. In such a case, we would have underestimated the effects of BTX administration.

For the BTX-injected masseter, EMG, mass and muscle-induced bite force all showed striking reductions, but differing in extent and in time course. EMG showed the earliest drop-off, already obvious a few days after injection, and the earliest recovery. The time course of EMG activity generally followed the expectations of the package insert that paralysis should be seen within 3 days and should be maximum at 1-2 weeks, lasting up to 3 months [45]. The lowest surface EMG readings (week 2, see Fig. 3) showed reductions of 60-70% from the non-injected side, but the more precise wire EMG results indicated that some regions of the masseter were reduced as much as 98% (middle location, calculated from Supplement B). The wire EMGs showed that the BTX was not distributed exactly where we placed it. The posterior masseteric location showed a delayed reduction in activity, and the close-by deep masseter was probably affected. It is unlikely that the injection site was incorrect because the EMG electrodes were inserted in the same locations as the injections; rather, BTX likely diffused along the fascial cleft that separates the posterior part of the superficial masseter from the deep masseter. Even at the end of the study, 11 weeks after BTX administration, some injected locations had subnormal activity levels.

Atrophy follows disuse, and thus minimum masseter mass probably did not occur until at least 3 weeks after injection. When we measured mass at 4 weeks, it was about 20% less than control values (Table 1). This moderate reduction in muscle mass falls within the 6%-40% range calculated from studies on rat masseter [20, 21] and mouse hindlimb [25], and as in these other studies may reflect edema, fibrosis or fat deposition [4, 45].

Masseter-induced bite force was dramatically decreased, 85% at week 3, and at week 7 was still 65% lower than control values (Supplement C, Fig. 6). Such poor force production is all the more remarkable because the pulse duration used (5 ms) should have been sufficient to stimulate muscle fibers directly rather than relying on the masseteric nerve. The loss of force capacity of the injected muscle was out of proportion to loss of muscle mass. A similar lack of correspondence between muscle mass and muscle force was noted in a study of rabbit quadriceps femoris, in which muscle mass was reduced 30-50% one month after injection, whereas knee extensor force had plummeted by 90-95% [44].

Human studies lack EMG observations during mastication and cannot measure muscle mass or force directly. However, muscle size can be estimated from imaging, and maximal voluntary clenching provides a measure of total bite force. Clinical studies consistently show thickness/volume reductions of approximately 30%, reaching nadir at 3 months post-BTX [4, 26, 27, 46], comparable to our 20% reduction at 1 month. EMG of the masseter during maximal clenching is reduced 70-75% at 2-4 weeks [28, 46], again comparable to our findings. However, bite force during clenching shows little reduction, only 3%-40% 2-3 weeks post-injection [26, 27, 30, 47]. The reason that clenching force remains relatively normal in spite of the fall-off of EMG presumably lies in the participation of non-injected muscles in this voluntary task.

4.3 Does BTX unload the TMJ and molar regions of the mandible?

In vivo bone strain for the molar part of the mandibular body of normal rabbits has been reported by others [32, 48]. The present data are consistent with these previous studies, both in absolute values and in the finding that the chewing side undergoes higher strain than the non-chewing side. In contrast to the mandibular body, normal levels of bone strain in the condylar neck of rabbits have not been measured previously, despite the common use of this species in TMJ research. A very careful computation based on detailed anatomical, EMG and jaw motion data [31] calculated that the loading on the chewing side condyle would be negligible during the power stroke, the entire reaction load being borne by the opposite, balancing side condyle. This calculation is in conflict with the present findings on the

condylar neck. Considering only the control saline animals, shear strains were similar on the two sides (approximately 350 $\mu\epsilon$), and on both sides the predominant principal strain was compressive and perpendicular to the tooth row. The best explanation for the discrepancy between these findings and the computation is that the transverse component of loading (which Weijs and Dantuma had been unable to partition between the two sides) caused a lateral bending of the condylar neck on the chewing side. The thin neck would have little resistance to transverse bending, and the resulting concavity on the lateral side would be read as compressive strain.

Because sample size for strain in the 4-week BTX animals was inadequate, it is best to treat the results as suggestions rather than findings. The decreased bone strain magnitudes at 4 weeks (20-50% lower than control values) suggest that BTX injection into the masseter did partially unload the mandible, as is usually assumed [7-9]. Importantly, the unloading appeared to be site-specific although not chewing side-specific. Four weeks after BTX, low strains and altered orientation characterized both sides of the molar region but only the injected-side condylar neck (Table 2). This finding directly relates to the fact that the masseter loads both sides of the molar region similarly, but differentially strains the condylar neck of its own side (Table 3).

Despite the sample size problem, the strain results support the conclusions drawn from muscle parameters, that compensation for the paralyzed masseter was incomplete, and that the force of mastication was reduced even though EMG had mostly returned at 4 weeks. Like the muscle parameters, bone strain was indistinguishable from normal at 12 weeks.

4.4 Patterns of bone loss in the mandible

The success of BTX treatment in decreasing force on the mandible raises the question of whether this non-weightbearing element undergoes bone loss. We found substantial bone loss 4 weeks after BTX injection, with a distribution that corresponded closely to the likely unloading pattern. Percent bone volume was decreased in molar regions of both sides, almost to the same extent (about 10%, Table 4), reflecting the fact that both sides of the mandibular body showed reduced strain. An even greater loss, affecting bone volume/area as well as percent bone volume/area, occurred in the injected-side condylar process, especially in the condylar head (40%, Table 4), whereas the non-injected side condylar process remained similar to controls in every parameter. The differential between sides reflects the fact that only the injected-side condyle was unloaded by BTX injection of the masseter. This finding negates a common assumption of previous studies that both sides would be affected [49].

If bone loss in the mandible can be ascribed directly to loss of bite force from the masseter, it should be possible to show specific correlations between muscle and bone parameters. A post hoc correlation matrix between masseter weight vs. bite force and bone parameters showed consistent results for the injected side of the BTX animals. BTX-injected masseter weight at 4 weeks was positively correlated with bite force ($r = 0.61$, $p = 0.15$), condylar head percent bone area ($r = 0.60$, $p = 0.07$) and trabecular thickness ($r = 0.58$, $p = 0.08$) and negatively correlated with trabecular separation ($r = -0.66$, $p = 0.04$). Lower but still positive correlations were seen for percent bone volume of the whole condylar process ($r = 0.48$) and of the molar region ($r = 0.14$). Similar comparisons for the non-injection side and for the saline animals showed no consistent pattern and no correlations approaching statistical significance. Thus this post hoc analysis supports the specific association of masseter paralysis and loss of trabecular bone in the same-side condyle.

The finding of bone loss in the mandible adds to evidence that this cranial bone responds readily to changes in muscular loading, notwithstanding the irrelevance of body weight. The

degree of osteoporotic change is consistent with studies on murine hindlimb after BTX injection of the calf musculature [13, 14, 25]. These studies found greater loss of bone in trabecular regions than in the cortices. The rabbit mandibular body is largely cortical, as is the condylar neck, but the condylar head is almost entirely trabecular, providing ample surface for resorption. The loss of trabecular bone from the condylar head was dramatic at 4 weeks (Table 5, Fig. 8B).

The condylar cartilage of the mandibular condyle and its fibrous covering function as a growth plate as well as an articulation, and the cartilage's capacity for growth remains long after puberty [50]. Thus it is theoretically possible for the condyle to increase in size even in adult animals. Reciprocally, under adverse biomechanical conditions resorption can occur, shrinking the condyle [51]. Such changes would be reflected in the overall tissue volume/area (first set of columns in Table 4). Our data suggest that at 4 weeks the non-injected side condyle was larger than the injected side condyle, which could reflect growth of this side, shrinkage of the injected side, or most likely, both.

4.5 Time course of recovery. Are there persistent effects?

For cosmetic usage on the muscles of the eye and forehead, the effect of BTX is temporary, necessitating repeat treatments roughly every 3-4 months [45]. It is not known whether these superficial facial muscles have regained fully normal activity, however. The clinical duration of BTX effect in masticatory muscles may be longer than 3-4 months. Although clenching force recovers quickly, this is probably due to other factors (see above). EMG activity in the human masseter returns after a few weeks but is not fully normal even at 6-7 months [28, 46]. Our findings indicate a similar delayed time course for masticatory recovery from BTX in non-humans. Rabbit EMG during chewing began to increase at week 3, but minor deficits were still present through week 9-11 (Figs. 3, 4).

Recovery from masseter atrophy is slow. Patients receiving BTX treatment for large masseters request re-injection at intervals of 5-19 months, not 3-4 [4, 5, 47]. Approximately half of the single-injection patient population still show masseter atrophy at 12 months [52], and repeated injections have a cumulative effect [5]. Rabbit masseters were still 8% lighter on average than the uninjected side at 12 weeks (Table 1). However, persistent atrophy seems to be a generalized BTX phenomenon, not unique to the jaw muscles, because mouse hindlimb muscle volume is also still reduced at 12-16 weeks [13, 15]. It is not clear why volume recovery is delayed, nor whether it is ever fully normalized. One possibility is that some neurons or muscle fibers do not survive the procedure, resulting in a permanent loss of muscle mass. Another possibility is alteration of fiber type; because BTX tends to make muscle fibers slower [53], they may become smaller. For cosmetic use to shrink large masseters, persisting atrophy is a positive outcome, but it casts doubt on the claim that BTX effects are strictly temporary.

In vivo bone strain at the molar region of the mandible was still reduced relative to saline controls on both sides of the jaw 12 weeks after BTX (Table 2), and strain on the injected side condylar neck tended to be low for working side cycles as well. Thus, like the other parameters, bone strain was more normal at 12 weeks than at 4, but lingering effects of BTX were still seen on the same-side condyle and both sides of the body. A strikingly similar pattern was seen for bone structure (Table 4). Although no longer statistically significant, the molar regions of both sides and the injected-side condylar processes still showed low bone volume and percent bone volume relative to saline controls.

The areal analysis of the condylar head shows that this important TMJ articulation remained osteoporotic 12 weeks after BTX administration. While the non-injected side was similar to controls, the injected side had 20% less percent bone area, a recovery of half of the 40%

difference seen at 4 weeks (Table 4). This region is entirely trabecular and hence very subject to resorption; moreover, once a trabecula is breached by resorption, it may be lost entirely if the remaining tissue is not load-bearing. This appeared to happen in the injected-side condyle. Whereas trabecular thickness and separation were notably improved at 12 weeks, the recovery of trabecular number was less (Table 5) and, under the rod model, did not even reach statistical significance. This persistent loss of trabecular bone, in contrast to the cortical bone of the condylar neck and molar region, is the same as the pattern observed after BTX use in the mouse hindlimb [13].

One point of great interest is the variation in recovery at 12 weeks. Some animals had hardly recovered at all while others had bone measurements in excess of controls. This can be seen in the unusually large standard deviations for condylar characteristics of BTX animals, affecting both sides at 12 weeks (Tables 4, 5). Other parameters also show very high variability in the BTX animals at 11-12 weeks, including masseter weight (Table 1), muscle activity (Fig. 4), muscle-induced bite force (Fig. 6) and condylar neck bone strain (Tables 2, 3). A post hoc correlation matrix found no pattern of relationship among muscle or bone parameters; the association between injected masseter weight and condylar trabecular bone, noted at 4 weeks, was no longer present. We conclude that the 12-week variability reflects individual reaction to the treatment. This is not unique to our study. Human subjects also vary greatly in their degree of recovery, and some parameters may eventually exceed their pre-BTX levels [27, 30].

5. Conclusions and clinical implications

After BTX paralysis of one masseter muscle, rabbits still masticate at typical rates and chew on both sides. Other than slight changes in the size and activity pattern of the medial pterygoid muscle, there is minimal compensation from other muscles of mastication. The uninjected side is not overloaded. Instead, muscle force declines for a considerable period of time, during which the masticatory bone strain is decreased on both sides of the tooth-bearing mandibular body and on the temporomandibular joint on the side of the injected masseter. These unloaded areas all lost bone after BTX treatment, whereas the normally strained temporomandibular joint on the uninjected side remained similar to saline controls. After a 12-week recovery period, masseter-induced bite force had returned to pre-injection levels, but the BTX-injected masseter was still smaller than the contralateral muscle, EMG activity was still affected, bone strain still tended to be low, and the bone content of the injected-side mandibular condyle was still severely depressed.

The translational significance of these findings may be considerable. Unloading of the mandible using BTX has been suggested as a technique to allow immediate placement of dental implants [6], but the present results show that such unloading might accelerate bone loss, a counterproductive outcome. An even greater concern is the persistent loss of bone microstructure at the mandibular condyle. Cosmetic BTX treatment of the masseters to change facial shape is performed on young individuals, often teenagers and usually female, and the treatment may be repeated many times. BTX is also used on patients with TMJ disorders who already have damaged condyles. In the long term, such usage may compromise the mechanical properties of the temporomandibular joint.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Fehlings D, Novak I, Berweck S, Hoare B, Stott NS, Russo RN. Botulinum toxin assessment, intervention and follow-up for paediatric upper limb hypertonicity: international consensus statement. *Eur J Neurol*. 2010; 17(Suppl 2):38–56. [PubMed: 20633178]
2. Friedman BC, Goldman RD. Use of botulinum toxin A in management of children with cerebral palsy. *Can Fam Physician*. 2011; 57:1006–73. [PubMed: 21918142]
3. Intiso D. Therapeutic use of botulinum toxin in neurorehabilitation. *J Toxicol*. 2012; 2012:802893. PMC3172973. [PubMed: 21941544]
4. Kim NH, Chung JH, Park RH, Park JB. The use of botulinum toxin type A in aesthetic mandibular contouring. *Plast Reconstr Surg*. 2005; 115:919–30. [PubMed: 15731696]
5. Kim NH, Park RH, Park JB. Botulinum toxin type A for the treatment of hypertrophy of the masseter muscle. *Plast Reconstr Surg*. 2010; 125:1693–705. [PubMed: 20517093]
6. Al-Muharrqi MA, Fedorowicz Z, Bareeq A, Al Bareeq R, Nasser M. Botulinum toxin for masseter hypertrophy. *Cochrane Database of Systematic Reviews*. 2009; 21 art. no. CD007510.
7. Schwartz M, Freund B. Treatment of temporomandibular disorders with botulinum toxin. *Clin J Pain*. 2002; 18:S198–203. [PubMed: 12569699]
8. Canter HI, Kayikcioglu A, Aksu M, Mavili ME. Botulinum toxin in closed treatment of mandibular condylar fracture. *Ann Plast Surg*. 2007; 58:474–8. [PubMed: 17452828]
9. Ihde SKA, Konstantinovic VS. The therapeutic use of botulinum toxin in cervical and maxillofacial conditions: an evidence-based review. *Oral Surg, Oral Med, Oral Pathol, Oral Radiol Endod*. 2007; 104:e1–e11. [PubMed: 17560141]
10. Clark GT, Stiles A, Lockerman LZ, Gross SG. A critical review of the use of botulinum toxin in orofacial pain disorders. *Dent Clin N Amer*. 2007; 51:245–61. [PubMed: 17185069]
11. Kim C, McCall WD, Kim YK, Chung JW. Effect of botulinum toxin on pressure pain threshold and EMG power spectrum of masseter muscle during sustained fatiguing contraction. *Am J Phys Med Rehabil*. 2010; 89:736–43. [PubMed: 20729652]
12. Ernberg M, Hedenberg-Magnusson B, List T, Svensson P. Efficacy of botulinum toxin type A for treatment of persistent myofascial TMD pain: a randomized, controlled, double-blind multicenter study. *Pain*. 2011 epub ahead of print.
13. Poliachik SL, Bain SD, Threet D, Huber P, Gross TS. Transient muscle paralysis disrupts bone homeostasis by rapid degradation of bone morphology. *Bone*. 2010; 46:18–23. [PubMed: 19857614]
14. Warner SE, Sanford DA, Becker BA, Bain SD, Srinivasan S, Gross TS. Botox induced muscle paralysis rapidly degrades bone. *Bone*. 2006; 38:257–64. [PubMed: 16185943]
15. Manske SL, Boyd SK, Zernicke RF. Muscle and bone follow similar temporal patterns of recovery from muscle-induced disuse due to botulinum toxin injection. *Bone*. 2010; 46:24–31. [PubMed: 19853070]
16. Manske SL, Boyd SK, Zernicke RF. Vertical ground reaction forces diminish in mice after botulinum toxin injection. *J Biomech*. 2011; 44:637–43. [PubMed: 21186027]
17. Rawlinson SCF, Mosley JR, Suswillo RFL, Pitsillides AA, Lanyon LE. Calvarial and limb bone cells in organ and monolayer culture do not show the same early responses to dynamic mechanical strain. *J Bone Miner Res*. 1995; 10:1225–32. [PubMed: 8585427]
18. Mavropoulos A, Odman A, Ammann P, Kiliaridis S. Rehabilitation of masticatory function improves the alveolar bone architecture of the mandible in adult rats. *Bone*. 2010; 47:687–92. [PubMed: 20601301]
19. Matic DB, Yazdani A, Wells RG, Lee TY, Gan BS. The effects of masseter muscle paralysis on facial bone growth. *J Surg Res*. 2007; 139:243–52. [PubMed: 17433895]

20. Tsai C-Y, Shyr Y-M, Chiu W-C, Lee C-M. Bone changes in the mandible following botulinum neurotoxin injections. *Eur J Orthodont*. 2011; 33:132–8.
21. Tsai C-Y, Huang R-Y, Lee C-M, Hsiao W-T, Yang L-Y. 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. [PubMed: 20189701]
22. 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. [PubMed: 20219321]
23. Tsai C-Y, Chiu WC, Liao Y-H, Tsai CM. Effects on craniofacial growth and development of unilateral botulinum neurotoxin injection into the masseter muscles. *Am J Orthod Dentofacial Orthop*. 2009; 135:142.e1–142.e6. [PubMed: 19201315]
24. Gross TS, Poliachik SL, Prasad J, Bain SD. The effect of muscle dysfunction on bone mass and morphology. *J Musculoskelet Neuronal Interact*. 2010; 10:25–34. [PubMed: 20190377]
25. Manske SL, Boyd SK, Zernicke RF. Muscle changes can account for bone loss after botulinum toxin injection. *Calcif Tissue Int*. 2010; 87:541–9. [PubMed: 20967431]
26. Ahn KY, Kim ST. The change of maximum bite force after botulinum toxin type A injection for treating masseteric hypertrophy. *Plast Reconstr Surg*. 2007; 120:1662–6. [PubMed: 18040203]
27. Yu C-C, Chen PK-T, Chen Y-R. Botulinum toxin A for lower facial contouring: a prospective study. *Aesthetic Plast Surg*. 2007; 31:445–51. [PubMed: 17674088]
28. Lee CJ, Kim SG, Kim YJ, Han JY, Choi SH, Lee SI. Electrophysiologic change and facial contour following botulinum toxin A injection in square faces. *Plast Reconstr Surg*. 2007; 120:769–78. [PubMed: 17700130]
29. Freund B, Schwartz M. The use of botulinum toxin for the treatment of temporomandibular disorder. *Oral Health*. 1998; 88:32–7. [PubMed: 9610334]
30. Freund B, Schwartz M, Symington JM. The use of botulinum toxin for the treatment of temporomandibular disorders: preliminary findings. *J Oral Maxillofac Surg*. 1999; 57:916–20. discussion 920-1. [PubMed: 10437718]
31. Weijjs WA, Dantuma R. Functional anatomy of the masticatory apparatus in the rabbit (*Oryctolagus cuniculus* L.). *Netherl J Zool*. 1981; 31:99–147.
32. de Jong WC, Koolstra JH, Korfage JAM, van Ruijven LJ, Langenbach GEJ. The daily habitual in vivo strain history of a non-weight-bearing bone. *Bone*. 2010; 46:196–202. [PubMed: 19857616]
33. van Wessel T, Langenbach GEJ, Kawai N, Brugman P, Tanaka E, Van Eijden TMGJ. Burst characteristics of daily jaw muscle activity in juvenile rabbits. *J Exp Biol*. 2005; 208:2539–47. [PubMed: 15961740]
34. Bredman JJ, Weijjs WA, Korfage HA, Brugman P, Moorman AF. Myosin heavy chain expression in rabbit masseter muscle during postnatal development. *J Anat*. 1992; 180:263–74. [PubMed: 1387129]
35. Widmer CG, Klugman D, English AW. Anatomical partitioning and nerve branching patterns in the adult rabbit masseter. *Acta Anat*. 1997; 159:222–32. [PubMed: 9605607]
36. Dechow PC, Carlson DS. A method of bite force measurements in primates. *J Biomech*. 1983; 16:797–802. [PubMed: 6643517]
37. Weijjs WA, Hillen B. Relationship between the physiological cross-section of the human jaw muscles and their cross-sectional area in computer tomograms. *Acta Anat*. 1984; 118:129–138. [PubMed: 6464637]
38. Parsa AA, Lye KD, Parsa FD. Reconstituted botulinum type A neurotoxin: clinical efficacy after long-term freezing before use. *Aesthetic Plast Surg*. 2007; 31:188–91. discussion 192-3. [PubMed: 17122996]
39. Micro-Measurements. Strain gage rosettes: selection, application and data reduction. Vishay Precision Group Tech Note TN-515. 2010:151–162.
40. Parfitt AM, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, Ott SM, Recker RR. Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. *J Bone Miner Res*. 1987; 2:595–610. [PubMed: 3455637]
41. Snedecor, GW.; Cochran, WG. Statistical Methods. 6th ed.. Iowa State Univ. Press; Ames, Iowa: 1967.

42. Yaraskavitch M, Leonard T, Herzog W. Botox produces functional weakness in non-injected muscles adjacent to the target muscle. *J Biomech.* 2008; 41:897–902. [PubMed: 18184613]
43. Tan EK, Jankovic J. Botulinum toxin A in patients with oromandibular dystonia: long-term follow-up. *Neurology.* 1999; 53:2102–7. [PubMed: 10599789]
44. Fortuna R, Vaz MA, Rehan Youssef A, Longino D, Herzog W. Changes in contractile properties of muscles receiving repeat injections of botulinum toxin. *J Biomech.* 2011; 44:39–44. [PubMed: 20828699]
45. Allergan Inc.. Package insert for Botox Cosmetic. 2002.
46. Kim JH, Shin JH, Kim ST, Kim CY. Effects of two different units of botulinum toxin type a evaluated by computed tomography and electromyographic measurements of human masseter muscle. *Plast Reconstr Surg.* 2007; 119:711–7. [PubMed: 17230111]
47. Kim KS, Byun YS, Kim YJ, Kim ST. Muscle weakness after repeated injection of botulinum toxin type A evaluated according to bite force measurement of human masseter muscle. *Dermatol Surg.* 2009; 35:1902–7. [PubMed: 19737290]
48. Weijs WA, de Jongh HJ. Strain in mandibular alveolar bone during mastication in the rabbit. *Arch Oral Biol.* 1977; 22:667–75. [PubMed: 272139]
49. Da Ré Guerra F, de Souza Pires IL, de Aro AA, Camargo LC, Pimentel ER, Palomari ET. Protocol on induction of TMJ articular disc degeneration in rats by utilization of botulinum toxin. *Arch Oral Biol.* 2010; 55:530–4. [PubMed: 20413106]
50. Luder, H-U. Postnatal Development, Aging, and Degeneration of the Temporomandibular Joint in Humans, Monkeys, and Rats. Center for Human Growth and Development, Univ. of Michigan; Ann Arbor, MI: 1996.
51. Zhao C, Kurita H, Kurashina K, Hosoya A, Arai Y, Nakamura H. Temporomandibular joint response to mandibular deviation in rabbits detected by 3D micro-CT imaging. *Arch Oral Biol.* 2010; 55:929–37. [PubMed: 20880518]
52. To EW, Ahuja AT, Ho WS, King WW, Wong WK, Pang PC, Hui AC. A prospective study of the effect of botulinum toxin A on masseteric muscle hypertrophy with ultrasonographic and electromyographic measurement. *Br J Plast Surg.* 2001; 54:197–200. [PubMed: 11254408]
53. Dodd SL, Selsby J, Payne A, Judge A, Dott C. Botulinum neurotoxin type A causes shifts in myosin heavy chain composition in muscle. *Toxicon.* 2005; 46:196–203. [PubMed: 15975617]

Highlights: Botulinum toxin in masticatory muscles (Rafferty et al.)

- A single injection of botulinum toxin into the rabbit masseter muscle caused atrophy that persisted for 12 weeks.
- Despite normal chewing, masseter-induced bite force was greatly reduced.
- *In vivo* bone strain recording indicated underloading of the injected-side mandibular condyle and both sides of the molar region.
- Substantial bone loss occurred in the underloaded regions, especially the injected-side condyle, which was still affected at 12 weeks.

Figure 1.
Experimental design.

Figure 2.

A. Superficial masseter muscle of the rabbit. The three locations shown as dots within circles are the points where BTX or saline was injected. These same three locations were the sites recorded during wire EMG sessions (anterior, middle and posterior superficial masseter). The x's within squares on the molar region and the condylar neck are the sites where strain gages were bonded to mandible for recording bone strain. Note partial removal of the deep masseter muscle over the neck of the condyle. **B.** Rabbit mandible. The shaded molar region and condylar process were removed for micro CT scanning.

Figure 3.

Average EMG amplitude of chewing bursts over time, measured using surface electrodes over the masseter muscle (data in Supplement A). Working (left chart) and balancing (right chart) cycles refer to whether the masseter was on the same side as the bolus or the opposite side, respectively. BL – baseline recording before injection of one masseter with either BTX or saline. The week 0 recording was made a few days after injection. Surface EMG was not performed during week 4. The uninjected masseters and the saline-injected masseters demonstrated similar EMG amplitudes throughout the entire period. EMG from the BTX-injected muscles dropped precipitously after injection and reached its lowest point during week 2. Levels remained low at least through week 10 when the injected muscle was on the working side but were normalized by week 5 when the bolus was on the opposite side. The asterisks indicate likely differences ($p < 0.05$) between the BTX-injected and the saline-injected muscles, and the double daggers indicate likely differences ($p < 0.05$) between the injected and uninjected sides of the BTX animals.

Figure 4.

Fine-wire EMG from BTX injected-side muscle locations during chewing at baseline (before injection, small-dotted horizontal line at 100%) and various post-injection time points. EMG amplitude is shown as a percentage of baseline (calculated from data in Supplement B). ASM (heavy solid line), MSM (medium solid line) and PSM (thin solid line) are the anterior, middle and posterior locations in the superficial masseter, all of which received BTX during week 0. The deep masseter (DM, dashed line) and medial pterygoid (MPT, heavy dotted line) muscles were not injected but may have received some BTX through diffusion. The error bars indicate one SE (not SD as elsewhere in this paper). All muscles tended to have reduced EMG 1-2 weeks after injection ($p < 0.05$ vs. baseline or controls for ASM and MSM). The injected ASM, MSM, and PSM remained low at 5-6 weeks ($p < 0.05$ for all three). No other differences reached $p < 0.05$.

Figure 5.

Raw recordings of fine-wire EMG from the same BTX group rabbit during working side chewing before (Baseline) and after (Week 1) BTX administration to the right superficial masseter muscle. Right (R) side muscles shown are the middle superficial masseter (R. MSM) and the medial pterygoid (R. MPT). On the uninjected side the left middle superficial masseter (L. MSM), posterior superficial masseter (L. PSM) and medial pterygoid (L. MPT) are shown. The dotted vertical lines show the onset of activity in the reference muscle (L. PSM) for each chewing cycle. The arrows indicate the onset of activity in the injected-side medial pterygoid (R. MPT). The loss of activity in the R. MSM after BTX injection is obvious. In addition, there was a change in the timing of contraction for the injected-side medial pterygoid. Before BTX, the R-MPT became active in synchrony with the other muscles, but after BTX it consistently started earlier. This change, seen consistently in the BTX animals, persisted until weeks 5-6 after BTX administration to the masseter.

Figure 6.

Relative bite force produced by BTX vs. saline-injected masseters. Each masseter was separately tetanized to produce a bite force at the incisors. The values shown are bite force produced by the injected muscle as a percentage of the uninjected muscle of the same animal (see Supplement C for absolute values). BTX or saline was injected after the baseline values were measured. The error bars represent one SD. Double asterisks denote $p \leq 0.002$.

Figure 7.

Compressive strains on the molar region and condylar neck during mastication. The upper figure shows averages from the saline controls, and the lower figure shows the injected side of BTX animals 4 weeks after the toxin was administered to the masseter muscle (data from Table 2). The length of the arrows is proportional to strain magnitude (note calibration arrow labeled $-400 \mu\epsilon$). Compared to saline controls and regardless of the side of chewing, strain was reduced and reoriented at both locations in the BTX sample.

Figure 8.

Coronal sections (reconstructed from micro CT scans) of pairs of mandibular condylar processes from representative rabbits. The injected-side condyle is shown on the left of each pair regardless of the actual side of injection. **A.** Four weeks after injection of saline into one masseter muscle. The dashed white outline shows the trabecular area analyzed as the condylar head for the lower part of Table 4 and for Table 5. The two condyles are similar in percent bone area. **B.** Four weeks after injection of BTX into one masseter. The injected-side condyle has less bone than the uninjected-side condyle. **C.** Twelve weeks after injection of BTX into one masseter. A differential between the two condyles in percent bone area is still apparent, particularly with regard to the greater trabecular separation on the BTX-injected side.

Table 1

Muscle weight: mean in grams [standard deviation]

		4 Weeks				12 Weeks			
		Side		Side		Side		Side	
Muscle	n	Injection	Non-injection	Paired <i>t</i>	n	Injection	Non-injection	Paired <i>t</i>	
BTX Masseter	10	6.54 [0.87]	8.01 [1.38]	<0.0001	9	7.73 [1.15]	8.38 [1.37]	0.02	
Saline Masseter	8	7.88 [1.03]	7.96 [1.00]	NS	9	8.41 [0.75]	8.30 [0.78]	NS	
<i>t-test between groups</i>		< 0.01	NS			NS	NS		
BTX Med Pterygoid	11	2.97 [0.44]	3.31 [0.44]	0.001	10	3.05 [0.54]	3.21 [0.58]	NS	
Saline Med Pterygoid	8	2.64 [0.66]	2.65 [0.70]	NS	10	3.03 [0.27]	3.06 [0.26]	NS	
<i>t-test between groups</i>		NS	0.02			NS	NS		
BTX Temporalis	9	1.34 [0.35]	1.39 [0.37]	NS	10	1.41 [0.19]	1.50 [0.26]	0.02	
Saline Temporalis	8	1.41 [0.30]	1.36 [0.23]	NS	10	1.46 [0.15]	1.51 [0.14]	NS	
<i>t-test between groups</i>		NS	NS			NS	NS		
BTX Lat Pterygoid	9	0.87 [0.12]	0.82 [0.13]	NS	10	0.87 [0.15]	0.86 [0.16]	NS	
Saline Lat Pterygoid	8	0.77 [0.11]	0.77 [0.10]	NS	10	0.85 [0.10]	0.84 [0.08]	NS	
<i>t-test between groups</i>		NS	NS			NS	NS		

Bone strain on the mandible during mastication: peak shear strain and orientation of minimum (compressive) principal strain (mean [standard deviation])

Table 2

Shear strain magnitude ^a				Orientation of compression ^a			
MOLAR REGION	n	Working side	Balancing side	n	Working side	Balancing side	n
Control ^b	10	408 µε [188]	306 µε [92]	10	46° [29]	8	62° [16]
4 week BTX							
injected side	1	318 µε	155 µε	1	105°	1	148°
non-injected side	3	187 µε [41]	215 µε [32]	3	52° [11]	3	105° [63]
12 week BTX							
injected side	3	213 µε [54]	141 µε [45]	3	47° [17]	5	72° [21]
non-injected side	6	279 µε [49]	176 µε [54]	6	55° [29]	4	49° [8]
CONDYLAR NECK							
Control ^b	9	385 µε [133]	343 µε [138]	9	89° [12]	7	96° [11]
4 week BTX							
injected side	1	302 µε	216 µε [123]	1	131°	4	126° [6]
non-injected side	6	374 µε [214]	376 µε [133]	6	89° [15]	3	98° [9]
12 week BTX							
injected side	3	245 µε [86]	348 [132]	3	87° [25]	5	102° [12]
non-injected side	6	610 µε [528]	371 µε [207]	6	84° [38]	3	90° [45]

^aThe magnitude of shear strain was calculated as maximum principal strain (usually tension) minus minimum principal strain (usually compression). Orientation of compression is relative to the occlusal plane (0° = 180°). 90° represents a vertical compression perpendicular to the occlusal plane. Angles less than 90° indicate a compressive orientation from anterosuperior to posteroinferior, and angles greater than 90° indicate a compressive orientation from posteroinferior to anterosuperior.

^bThe control sample combined 4-week and 12-week endpoints of the saline group, and both injected and non-injected sides, as these did not differ.

Table 3

Strain on the mandible from tetanus of the BTX-injected masseter vs control masseters; peak shear strain and orientation of minimum (compressive) principal strain (mean [standard deviation])

Strain on side of contracting masseter				Strain opposite to contracting masseter				
	<i>n</i>	Shear strain magnitude ^a	<i>n</i>	Orientation of compression ^a	<i>n</i>	Shear strain magnitude ^a	<i>n</i>	Orientation of compression ^a
MOLAR REGION								
Control muscles ^b	14	241 με [171]	12	16° [30] ^c	14	173 με [156]	12	145° [35]
Injected Muscle								
BTX 4 weeks	1	112 με	1	97°	2	13 με [8]	--	---
BTX 12 weeks	3	111 με [77]	2	5° [48]	4	230 με [375]	3	12° [3]
CONDYLAR NECK								
Control muscles ^b	15	367 με [246]	15	73° [30]	15	207 με [200]	15	126° [38]
Injected muscle								
BTX 4 weeks	2	22 με [23]	--	--	2	21 με [18]	--	--
BTX 12 weeks	5	481 με [595]	5	91° [32]	4	192 με [192]	3	69° [45]

^aSee Table 2 for definitions. Orientation cannot be determined accurately when strain magnitudes are very low, as was usually the case for BTX-injected muscles at 4 weeks.

^bControl muscles are from saline animals at both 4-week and 12-week endpoints and include injected and non-injected muscles.

Table 4

Micro CT analysis of bone quantity in the molar and condylar regions of the mandible: mean [standard deviation]

		Tissue Volume (mm ³)				Bone Volume (mm ³)				% Bone Volume	
		n	Injection	Non-Injection	Paired t-test	Injection	Non-Injection	Paired t-test	Injection	Non-Injection	Paired t-test
Molar region (body and alveolar bone) volume											
4 Weeks											
BTX	9	234 [18]	237 [20]	NS	125 [8]	132 [10]	0.06	53.5 [2.1]	55.8 [2.4]	0.04	
Saline	7	229 [32]	234 [36]	NS	138 [21]	141 [22]	NS	60.3 [3.1]	60.0 [3.2]	NS	
t-test		NS	NS		NS	NS		0.0001	0.01		
12 Weeks											
BTX	10	238 [16]	241 [15]	NS	136 [8]	141 [10]	0.01	57.2 [4.6]	58.5 [3.0]	NS	
Saline	9	237 [27]	228 [40]	NS	142 [11]	142 [14]	NS	60.3 [3.3]	59.8 [3.3]	NS	
t-test		NS	NS		NS	NS		NS	NS		
Condylar process (head and neck) volume											
4 Weeks											
BTX	11	112 [16]	132 [24]	0.001	49 [10]	71 [17]	<0.0001	43.6 [6.4]	53.1 [6.3]	<0.0001	
Saline	9	122 [22]	124 [18]	NS	69 [14]	69 [10]	NS	56.3 [4.3]	55.6 [4.3]	NS	
t-test		NS	NS		0.001	NS		<0.0001	NS		
12 Weeks											
BTX	10	124 [20]	129 [15]	NS	70 [16]	76 [15]	0.04	56.0 [6.3]	59.0 [7.6]	0.02	
Saline	9	124 [21]	122 [21]	NS	75 [13]	77 [11]	NS	60.8 [4.6]	63.6 [5.9]	NS	
t-test		NS	NS		NS	NS		NS	NS		
Condylar head area											
4 Weeks											
BTX	11	8.0 [2.2]	9.3 [2.8]	0.02	3.0 [1.0]	6.1 [1.4]	0.0001	39.1 [11.2]	67.5 [10.8]	<0.0001	
Saline	9	7.9 [2.7]	8.1 [3.4]	NS	5.1 [1.7]	5.4 [2.5]	NS	65.4 [10.7]	64.9 [6.8]	NS	
t-test		NS	NS		0.003	NS		<0.0001	NS		
12 Weeks											

	n	Tissue Volume (mm ³)			Bone Volume (mm ³)			% Bone Volume		
		Injection	Non-Injection	Paired <i>t</i> -test	Injection	Non-injection	Paired <i>t</i> -test	Injection	Non-Injection	Paired <i>t</i> -test
BTX	9	8.2 [2.4]	8.8 [3.0]	0.06	4.4 [1.6]	5.6 [2.2]	0.04	54.2 [10.7]	64.0 [11.8]	0.03
Saline	9	8.5 [1.9]	8.8 [3.4]	NS	5.8 [1.3]	6.0 [2.2]	NS	67.8 [3.1]	68.0 [3.6]	NS
<i>t</i> -test		NS	NS		NS	NS		0.002	NS	

Table 5

Trabecular bone of condylar head sections: mean [standard deviation]^a

Treatment	n	Trabecular thickness (µm)			Trabecular separation (µm)			Trabecular number/mm		
		Injection	Non-Injection	Paired <i>t</i> -test	Injection	Non-injection	Paired <i>t</i> -test	Injection	Non-Injection	Paired <i>t</i> -test
4 Weeks										
BTX	11	34.1 [3.3]	39.5 [2.6]	0.001	57.6 [20.1]	20.2 [10.1]	0.0001	11.3 [2.2]	17.0 [1.9]	<0.0001
Saline	9	38.6 [3.0]	38.5 [3.0]	NS	21.6 [10.6]	21.3 [5.0]	NS	16.9 [2.3]	16.8 [1.0]	NS
<i>t</i> -test		0.006	NS		0.0001	NS		<0.0001	NS	
12 Weeks										
BTX	9	38.5 [2.5]	40.0 [2.9]	NS	35.3 [16.7]	24.1 [12.1]	0.01	14.0 [2.2]	15.9 [2.0]	0.01
Saline	9	40.9 [1.2]	41.0 [1.3]	NS	21.1 [4.1]	19.2 [2.6]	NS	16.2 [0.9]	16.7 [0.5]	NS
<i>t</i> -test		0.02	NS		0.02	NS		0.01	NS	

^aCalculations assumed a parallel plate model for trabecular architecture [40]. Trends were the same for the rod model calculations, but absolute values were different and statistical significance was slightly lower.