Botulinum toxin in masticatory muscles of the adult rat induces bone loss at the condyle and alveolar regions of the mandible associated with a bone proliferation at a muscle enthesis

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A R T I C L E   I N F O

Article history:
Received 23 November 2014
Revised 31 March 2015
Accepted 31 March 2015
Available online 7 April 2015
Edited by David Fyhrie

Keywords:
Botulinum toxin
Mandible
Dissuse osteoporosis
Masticatory muscles
Torus mandibularis

A B S T R A C T

In man, botulinum toxin type A (BTX) is injected in masticatory muscles for several indications such as trismus, bruxism, or masseter hypertrophy. Bone changes in the mandible following BTX injections in adult animal have therefore became a subject of interest. The aim of this study was to analyze condylar and alveolar bone changes following BTX unilateral injections in masseter and temporal muscles in adult rats. Mature male rats (n = 15) were randomized into 2 groups: control (CTRL; n = 6) and BTX group (n = 9). Rats of the BTX group received a single injection of BTX into right masseter and temporal muscles. Rats of the CTRL group were similarly injected with saline solution. Rats were sacrificed 4 weeks after injections. Masticatory muscles examination and microcomputed tomography (microCT) were performed. A significant difference of weight was found between the 2 groups at weeks 2, 3 and 4 (p < 0.05). Atrophy of the right masseter and temporal muscles was observed in all BTX rats. MicroCT analysis showed significant bone loss in the right alveolar and condylar areas in BTX rats. Decrease in bone volume reached −20% for right alveolar bone and −35% for right condylar bone. A hypertrophic bone metaplasia at the digastric muscle enthesis was found on every right hemimandible in the BTX group and none in the CTRL group. BTX injection in masticatory muscles leads to a significant and major mandible bone loss. These alterations can represent a risk factor for fractures in human. The occurrence of a hypertrophic bone metaplasia at the Mus Digastricus enthesis may constitute an etiological factor for tori.

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Introduction

Type A botulinum toxin (BTX) is a bacterial metalloprotease produced by Clostridium botulinum. It is a neurotoxin that causes specific inhibition of the neurotransmitter release in cholinergic nerve terminals. Acetylcholine vesicles are blocked at the presynaptic membrane of neuromuscular junctions because BTX degrades the SNAP-25 protein required for vesicle fusion and release of acetylcholine at the axon end [1]. This leads to a transient muscle paralysis which is fully reversible –3]. BTX injection in the Masticatory muscles (Mus Masseter and/or Mus Temporalis) for several indications such as trismus, bruxism, masticatory myalgia, temporomandibular joint disorders or masseter hypertrophy [7–10]. It is also used in facial injections (subcutaneous, intraglandular or intramuscular); the main indications being sialorrhoea, blepharospasm, hemifacial spasm and aesthetic use [7,8,11]. There are level 1 or 2 evidences supporting the efficacy of BTX in many of those treatments [7,12]. Repeated injections are needed in many indications. Side effects are rare and reversible: non-desirable focal facial muscle paralysis by toxin diffusion and complications at the injection site (bruising, pain, and edema) [13–16].

The mandible is a non-weight bearing bone which is stimulated by masticatory muscles. It is composed of alveolar and basal bone. Teeth roots are anchored into alveolar bone by the periodontal ligament. Alveolar bone has a high plasticity and is remodeled at a high rate. Its mechanical stimulation during mastication is essential in keeping the teeth and underlying bone healthy. Loss of teeth leads to an irreversible alveolar bone resorption [17,18]. Alveolar bone loss is also found in several metabolic bone diseases or due to glucocorticoid treatment [19–21]. However, trabecular bone is also present in other parts of the mandible such as the condylar process. Fractures of this area are also observed in clinical practice and may occur in osteoporosis [22,23].

The aim of the present study was to evaluate bone changes following a unilateral BTX injection in the right masseter and temporalis muscles in adult rats. Two areas were selected and analyzed by microcomputed tomography (condylar and alveolar bone) 4 weeks after injection.
Material and methods

Animals and experimental procedure

Eighteen week-old male Sprague–Dawley rats (n = 15), weighing 595 ± 41 g, were used for the study (Janvier-Labs, Le Genest-Saint-Isle, France). They were acclimated for two weeks to the local vivarium conditions (24 °C and 12 h/12 h light dark cycle) where they were given standard laboratory food (UAR, Villemaison-sur-Orge, France) and water ad libitum. Rats were randomized into 2 groups: control group (CTRL, n = 6) and BTX-injected group (BTX, n = 9). Rats from the BTX group were anesthetized with isoflurane and injected intramuscularly with 2U of type A BTX: 1 U in 0.2 ml in the Mus Masseter and 1 U (in 0.2ML) in the Mus Temporalis (Botox®, Allergan Inc., Irvine, CA, USA). Three points of injections for each Mus Masseter and two for each Mus Temporalis were necessary. Rats of the CTRL group were similarly injected with equivalent volume of saline solution. Rats were weighted weekly and were sacrificed 4 weeks after injection by CO2 inhalation. Facial skin was carefully dissected and removed to perform visual examination of right and left masticatory muscles. Hemimandibles were then dissected, defleshed and fixed in formalin until use.

Animal care and experimental protocols were approved by the French Ministry of Research and were done in accordance with the institutional guidelines of the French Ethical Committee (protocol agreement number 01732.01) and under the supervision of authorized investigators.

Microcomputed tomography (microCT)

MicroCT of right and left hemimandibles was performed using a Skyscan 1172 X-ray computerized microtomograph (Bruker microCT, Kontich, Belgium) equipped with an X-ray tube working at 70 kV/100 μA. Bones were placed in plastic tubes filled with water to prevent desiccation. The tubes were fixed on a brass stub with plasticine. Analysis was done with a pixel size corresponding to 10.5 μm; the rotation step was fixed at 0.25° with a 0.5 mm aluminum filter. For each hemimandible, a stack of 2D-sections was obtained and reconstructed using NRecon software (Bruker) and analyzed with CTan software (Skyscan, release 1.13.11.0). Frontal and sagittal sections of alveolar regions and frontal sections of condylar regions were then obtained from 3D models to perform measurements.

Alveolar bone measurements

Because the amount of alveolar and condylar bone is very limited in rodents, measurement of the trabecular bone volume could not be done in 3D and its equivalent (fraction of the tissue area occupied by trabecular bone — B.Ar/T.Ar, expressed in %), was obtained on 2D sections as previously reported [19]. Measurement of B.Ar/T.Ar was done on sections of right and left hemimandibles using ImageJ 1.49c software. Condylar B.Ar/T.Ar was measured using 10 serial frontal sections obtained in the middle of the condylar head (Fig. 1A). Alveolar B.Ar/T.Ar was measured using 5 frontal sections and 1 sagittal section of alveolar region (Fig. 1B–C). Measurements were averaged for each bone.

Cortical bone measurements

Measurements of the mandibular cortical thickness were performed on 2D frontal sections of the right and left hemimandibles (taking the distal root of the first molar as a reference point) using CTan. Eight measures were done (4 for the lingual side and 4 for the vestibular side) and averaged to provide the lingual (LCt.Th, in μm) and vestibular (VCt.Th, in μm) cortical thickness (Fig. 1B).

Analysis of mandibular 3D porosity by a vector projection algorithm

Binarization of stacks of 2D sections was performed using CTan software and transferred to a lab-made software written in Matlab (Math Works, Natick, MA) release 7.10. The algorithm has been extensively described elsewhere [24]. Briefly, on the binarized images, porosity was visible in white and bone in black. For each binarized image of the stack, the pores which belong to the same image column received the same pseudo-color according to a look up table (LUT). The same color was applied on the image boundary and on a frontal plane image which was constructed line by line from all images of the microCT stack. A 3D model was reconstructed from the subsequent colorized images with VG Studiomax (Volume Graphics GmbH, Heidelberg, Germany). The frontal plane image was saved with the colorized LUT and analyzed by the FracLab plug-in module developed for ImageJ, to obtain the box plot fractal dimension Df (of the frontal plane image) [25,26].

Histology

Osteotomy of the hemimandibles was performed in order to isolate the molar area. Samples were then decalcified using Decalcifier II® (Surgipath®, Leica Biosystems, Richmond Inc., USA) during 8 days. The specimens were embedded in paraffin. Serial frontal sections were performed (6 μm in thickness) and stained with HPS (hematoxylin, phloxine saffron).

Statistical analysis

Statistical analysis was performed using the Systat statistical software release 13.0 (Systat Software Inc., San Jose, CA). All data were expressed as mean ± standard error of the mean (SEM). Differences among groups were analyzed by a non-parametric ANOVA (Kruskall–Wallis) and between groups by the Mann and Whitney’s U test. Data
from right and left hemimandibles were compared using a paired t-test. Differences were considered significant when \( p < 0.05 \).

**Results**

**Body weight and anatomic muscle examination**

No significant difference in body weight was found within each group during the time course of the study. However, a significant difference was found between the 2 groups at weeks 2, 3 and 4 (\( p < 0.05 \)) (Fig. 2). The weight loss was \(-15 \pm 11.17 \) g (i.e. \(-2.5\% \) of initial weight) in the BTX group. All rats of the BTX group presented a masseter and temporal atrophy at the injected side (Fig. 3). No differences were noticed in the CTRL group.

**MicroCT analysis of bone effects of BTX injection**

Bone loss was clearly seen on the right side of alveolar bone in the BTX group with less trabeculae and wider marrow cavity (Figs. 4A–B). No difference was noted when comparing both sides of the CTRL group. Trabecular bone loss was also clearly seen on the right side of condylar bone in the BTX group (Figs. 4C–D) with a reduced trabecular density. No differences were noted when comparing both sides of the CTRL group.

Of considerable interest was the occurrence of a hypertrophic bone proliferation at the enthesis of Mus Digastricus on the right hemimandible. This proliferation was observed in every rats of the BTX group but was not encountered on the non-paralyzed left side (Figs. 4B and 5B). No such proliferation occurred in the CTRL group. These bony proliferations appeared composed of thin trabeculae which evoked a bone metaplasia.

MicroCT measurements are summarized in Table 1. A marked decrease in alveolar B.Ar/T.Ar (reaching \(-20\% \)) was observed at the right vs. left hemimandible (\( p < 0.0001 \)) in the BTX group. Similarly, \( a - 35\% \) reduction in condylar B.Ar/T.Ar occurred at the right vs. left hemimandible (\( p < 0.0001 \)). The BTX group had a significantly lower condylar B.Ar/T.Ar and alveolar B.Ar/T.Ar at the right side compared to the CTRL group (\( p < 0.05 \)). There were no significant differences between left sides of the BTX group and both sides of the CTRL groups for all parameters.

Cortical bone thickness was not significantly reduced on the right side of the BTX group at both lingual and vestibular sites.

**Analysis of mandibular porosity by vector analysis**

Porosity could be well evidenced on the frontal plane and at the surface of the 3D models (Figs. 5C–D). The most porous areas appeared as red-orange and yellow areas (hot colors) while the blue areas indicate a low porosity according to the LUT selected. The area comprising the roots and the alveolar bone was selected by hand drawing of a ROI on the frontal plane images. The lower limit corresponded to the upper part of the incisor socket. No difference was noted morphologically between the left and right side in the CTRL group. On the other hand, the amount of hot areas was increased on the right side of BTX group. Quantitative analysis confirmed that D\(_{2}\) was significantly increased in the BTX right side versus the left side and also versus the CTRL sides (\( p < 0.02 \)) (Table 1).

**Histological analysis**

Study of the hypertrophic bone proliferation at the enthesis of Mus Digastricus evidenced bone metaplasia with woven bone in active remodeling. Numerous osteoblast alignments were seen together with large osteoclasts (Fig. 6). Fibers of the digastric muscle were seen anchored at the surface of this bony proliferation. The separation between normal cortical bone and woven bone was clearly identified. Cortical bone appeared made of lamellar bone in CTRL group. (See Fig. 7.)

**Discussion**

The present study was designed to determine the effect of motor denervation induced by Clostridium botulinum toxin serotype A on the mandibular bone of the rat. In our study, amyotrophy was anatomically evidenced by the naked eye and no attempt was done to quantify muscle loss after dissection. Amyotrophy after BTX injection has been reported in a large number of papers [4,27–31]. It is due to a complete motor denervation starting between 2 and 5 days after injection; paralysis is reversible in 4 to 6 weeks. BTX injection is known to reduce the thickness and weight of muscles [32]. It is likely that the failure to gain weight in BTX rats did not reflect the amyotrophy of Mus Masseter and Mus Temporalis but was provoked by a slightly reduced food intake due to difficulty in chewing.

Condylar and molar areas of the injected side are under-loaded after BTX injections [27]. Although the gain (or loss) of body weight was not significant in each group, the weight loss reached \(-2.5\% \) of initial weight in the BTX group and differences with the CTRL group became significant after 2 weeks. This indicates that animals did not suffer...
from food starvation after BTX injection. Surprisingly, weight loss has
not been reported in other studies with unilateral or even bilateral
BTX injections in *Mus Masseter* and/or *Mus Temporalis* in growing or
adult animals (rats or rabbits) [27–31,33]. Rabbits are reported to
keep chewing at an unaltered rate despite a significant functional deficit
of injected muscles [27]. Injection of BTX in other types of muscle (such
as the *Mus Quadriceps femoris*) did not affect the body weight [6,34], so
the effect noted in the present study appears specific to the site of injec-
tion. In rabbits with BTX injections in right *Mus Masseter*, the muscle
was found significantly 15–18% lighter than the contralateral muscle
and *Mus Pterygoideus medialis* of the non-injected side was found sig-
ificantly 25% heavier than contralateral muscles, this tends to indicate a compensatory mechanism [27,29]. In growing rats with bilateral BTX
injections and muscle volume measurements by plethysmometry, the
injected masseter volume was found 31% lower if injected alone and
47% lower if *Mus Temporalis* was injected at the same time [28]. In
human, it has been shown that *Mus Masseter* loses 30% of its volume
3 months after a BTX injection [35].

In the present study, 1 U of BTX was injected in the 2 main masticatory muscles on the right side. We chose to use 3 points of injection in the *Mus Masseter* and 2 points of injection in the *Mus Temporalis* to ensure complete diffusion in the entire muscle; this is similar to BTX injection in humans for clinical purposes. We chose an injection volume of 0.2 ml for 1 U of BTX. In human, the dilution of BTX is 5 U in 0.2 ml of saline and usually 30 to 50 U are injected per each masticatory muscle. We chose to use 1 U of BTX per each muscle and a less concentrated solution because of the smaller muscle volume in rats compared to humans. One of the main interests of the BTX model is that the paralyzed side can be compared with the contralateral side; in other words, the animal is its own control [5,34]. In the literature, the doses used are comprised between 1 and 7.5 U per masticatory muscle in the rat, with comparable results [27–31,33].

In order to assess bone loss, microCT and subsequent image analysis treatments were used. Mandibular bone mineral density by dual energy X-ray densitometry was not measured in the present study because the method is not reliable and reproducible enough at the mandible with devices available for clinical purposes, even with the use of software for small animals [6,36]. In the present study, a major decrease of B.Ar/T.Ar was found at condylar and alveolar bone. However, due to the complexity of the alveolar region (presence of the molar roots, proximity of the incisor), it was not possible to determine a volume of interest in 3D with the technique offered by the software provided by
the microCT manufacturer and the 2D measurements are favored in this location for rodents [19]. Our results fit well with other studies using BTX injections in masticatory muscles of adult animals [27,31]. In the rabbit, the decrease in alveolar B.Ar/T.Ar reached −6% for the BTX-paralyzed side and −12% for condylar bone vs. control animals; however, the decrease was only −2% for alveolar bone and −9% for condylar bone when compared to the uninjected side [27]. In the present study, bone loss was higher (−20%) for alveolar bone and (−35%) for condylar bone, however, this might reflect differences in morphometric evaluation. A similar decreased B.Ar/T.Ar was reported in an adult rat model with mandibular hypofunction due to a soft diet [37]. The vector projection algorithm used here appeared interesting in the evaluation of the alveolar bone. The method allows evaluation of a complex volume of interest and provides quantitative measurements of the complexity of bone loss in the alveolar area; the roots had no influence on the measurements. We have previously reported that the

Table 1

<table>
<thead>
<tr>
<th>Bone morphometric parameters.</th>
<th>CTRL Left</th>
<th>CTRL Right</th>
<th>BTX Left</th>
<th>BTX Right</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alveolar B.Ar/T.Ar (%)</td>
<td>59.66 ± 1.87</td>
<td>58.92 ± 2.85</td>
<td>60.29 ± 1.96</td>
<td>40.27 ± 1.65&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Condylar B.Ar/T.Ar (%)</td>
<td>67.32 ± 3.42</td>
<td>66.58 ± 3.81</td>
<td>69.62 ± 1.21</td>
<td>35.22 ± 2.49&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>LCL.Th (μm)</td>
<td>512 ± 13</td>
<td>494 ± 15</td>
<td>518 ± 19</td>
<td>483 ± 25</td>
</tr>
<tr>
<td>VCL.Th (μm)</td>
<td>506 ± 18</td>
<td>518 ± 11</td>
<td>504 ± 19</td>
<td>483 ± 22</td>
</tr>
<tr>
<td>D&lt;sub&gt;f&lt;/sub&gt; frontal plane</td>
<td>1.416 ± 0.010</td>
<td>1.420 ± 0.018</td>
<td>1.419 ± 0.009</td>
<td>1.474 ± 0.009&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
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<sup>a</sup> Indicates a significant difference between the right vs. the left side.

<sup>b</sup> Indicates a significant difference vs. CTRL.

![Fig. 5. MicroCT analysis of the hemimandibles of rat with BTX paralysis of Mus Masseter and Mus Temporalis on the right side. A) General view from the lingual side of the left hemimandible. B) Similar view of the right hemimandible. The green arrow identifies the hypertrophic bone proliferation at the Mus Digastricus enthesis. Analysis of porosity by vector analysis on microCT sections in the molar region of the same bones C) left hemimandible, D) right hemimandible. The red pseudo color of alveolar bone indicates higher bone porosity according to the LUT. Note the considerable increase in hot colors (red-orange-yellow in the alveolar area of the right side.](image-url)
The fractal dimension measured on the frontal plane was well correlated with the 3D porosity in an in vitro model [24]. In addition, the method allows a mapping of porosity at the surface of the mandibular bone and evidenced the differences between the right and left sides in these animals.

Masticatory function is significantly decreased in human after BTX injection into masticatory muscles; masticatory efficiency recovers completely after 12 weeks [38]. All these results show the importance of masticatory function on alveolar and condylar bone remodeling and architecture. It is noticeable that in our study, as in others, the percentages of bone loss were higher in condylar bone compared to alveolar bone. This fact seems consistently described. Cortical bone thickness was not significantly reduced in our study whereas it was significantly lower in similar models by others [27,28,31]. A possible explanation could be that this occurred only in growing animals. Another explanation could be differences with other studies in the localization and number of measuring sites of cortical bone. A last explanation is that a 4 weeks endpoint is insufficient to develop a cortical bone loss due to the lower remodeling rate in this bony envelope. Longer studies are necessary to evaluate the cortical bone loss which is an important parameter to assess since it is known that cortical changes increase fracture risk in humans [39].
A relevant finding was that hypertrophic bone proliferation made of metaplastic bone developed on the paralyzed side at the mandibular Masseter muscles. An increased activity of Masseter muscle on the paralyzed side probably lead to a local increase of mechanical strains at the mandible which compensates the loss of activity of the temporal and masseter muscles. An increased muscle activity is known to stimulate bone remodeling leading to an increased bone mass [41]. It has also been shown that it induces the development of woven bone [42]. In rabbits injected by BTX in Masseter muscle, a compensating hypertrophy of Masseter muscles mimics a torus mandibularis. Although the pathophysiology of tori is not fully understood, a relationship between tori and a disequilibrium of occlusal forces is hypothesized [43].

Differences between our model and others of the literature (failure to gain weight in growing animals, more important percentages of bone loss, hypertrophic bone metaplasia) were found. Those bone alterations, if they happened equally in human, could constitute a major risk factor for fractures, especially in patients receiving repeated BTX injections in masticatory muscles. This remains to be studied in human since there are many differences between rat and human masticatory functions (masticatory apparatus, bone volume, dosage of BTX). One single study in the literature reports mandibular bone assessment (cortical thickness, mandibular thickness, and total mandibular volume) following BTX unilateral single injections in masseter in human with no significant change [35]. Furthermore, future works shall aim at assessing bone loss in human receiving those treatments and at assessing minimal periods of time between injections in human in order to avoid potential increased risk fracture.

Conflicts of Interest

The authors declare that they have no conflicts of interest concerning this article.

Acknowledgments

Authors are greatly indebted to the SCAHU (Service commun d’animalerie hospitalo-universitaire) of Angers, especially to P. Legras and J. Roux for their help with the animal care. Our vector analysis software (VECTOPOR) is now licensed by the APP (Agence de Protection des Programmes). JDKD IDDNI.FR.001.150014.000.S.P.2015.000.10000 received a scholarship from the ARS (Agence Régionale de Santé) “Pays de la Loire”.

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