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LA TOXINE BOTULIQUE INJECTEE DANS LES MUSCLES MASTICATEURS CHEZ LE RAT ADULTE ENTRAINE UNE PERTE OSSEUSE MANDIBULAIRE CONDYLIENNE ET ALVEOLAIRE ASSOCIEE A LA PROLIFERATION OSSEUSE D'UNE ENTHESE

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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novembre 2014

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LIST OF ABBREVIATIONS

BV/TV: Trabecular bone volume

BTX: Botulinum toxin

CTRL: Control

Ct.Th: Cortical bone thickness

Df: Fractal dimension

LCt.Th: Lingual cortical bone thickness

LUT: Look up table

MicroCT: Microcomputed tomography

Mf: Muscular fibers

ROI: Region of interest

SEM: Standard error of the mean

VCt.Th: Vestibular cortical bone thickness

PLAN

1. 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 in a few months [1-3]. BTX injection in the *Mus Quadriceps femoris* of rodents is now well-known to create a model of tibial and femoral bone loss by immobilization which is easily quantified by reduction of the trabecular bone volume and cortical thickness [4-6].

In human, BTX injections are used in 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.

2. MATERIAL AND METHODS

2.1. Animals and experimental procedure

Eighteen weeks-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, Villemoison-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: 1U in the *Mus Masseter* and 1U 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 CO_2 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.

2.2. 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.

2.2.1 Alveolar bone measurements

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

2.2.2 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).

2.3. Analysis of mandibular 3D porosity by a vector projection algorithm

Binarization of stacks of 2D sections was performed using CTan software (Skyscan) 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 plugging developed for ImageJ, to obtain the box plot fractal dimension D_f (of the frontal plane image) [25, 26].

2.4. 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).

2.5. 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 $p<0.05$.

3. RESULTS

3.1. Body weight and anatomic muscle examination

The change in body weight was insignificant in rats of the CTRL and BTX groups during the time 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 ± 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 amyotrophy at the injected side (Fig. 3). No differences were noticed in the CTRL group.

3.2. 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 (Fig. 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 (Fig. 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 (Fig. 4B and 5B). No such proliferation occurred in the CTRL group. These bony proliferations appeared composed of thin trabeculae which evocated a bone metaplasia.

MicroCT measurements are summarized in Table 1. A marked decrease in alveolar BV/TV (reaching -20%) was observed at the right *vs.* left hemimandible ($p<0.0001$) in the BTX group. Similarly, a -35% reduction in condylar BV/TV occurred at the right *vs.* left hemimandible ($p<0.0001$). The BTX group had a significantly lower condylar BV/TV and alveolar BV/TV 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.

3.3. Analysis of mandibular porosity by vector analysis

Porosity could be well evidenced on the frontal plane and at the surface of the 3D models (Fig. 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 morphological difference was noted between the left and right sides 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_f was significantly increased in the BTX right side versus the left side and also versus the CTRL sides ($p<0.02$) (table I).

3.4. 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.

4. 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 at naked eye and no attempt was done to quantify muscle loss after dissection. Amytrophy 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 significant loss of body 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. It has been shown that condylar and molar areas of the injected side are under loaded after BTX injections using analysis of bone strains during mastication [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 w. 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 of 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 injection. 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 significantly 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. 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 to 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 BV/TV 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 in well with other studies using BTX injections in masticatory muscles of adult animals [27, 31]. In the rabbit, the decrease in alveolar BV/TV 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). A similar decreased BV/TV 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; tooth roots had no influence on the measurements. We have previously reported that 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.

It has been shown that masticatory function was significantly decreased after BTX injection into masticatory muscles and that masticatory efficiency only completely recovered after 12 weeks, in human [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 constantly 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. This is an important parameter to assess since it is known that cortical bone alteration increases a lot fracture risks in human [39].

Of the upmost importance was that hypertrophic bone proliferation made of metaplastic bone developed on the paralyzed side at the mandibular *Mus Digastricus* enthesis in every animal of the BTX group. *Mus Digastricus* is an accessory masticatory muscle and a jaw-opener [40]. An increased activity of *Mus Digastricus* on the paralyzed side probably leads 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 *Mus masseter*, a compensating hypertrophy of *Mus Medial pterygoid* (another jaw-closer main masticatory muscle) has been reported [27]. However no such hypertrophic bone metaplasia was reported at the muscle enthesis (the *pterygoid fossa*, on the vestibular side of the mandible) and we also did not find bone changes in this location on our microCT sections. Such a hypertrophic metaplasia mimics a *torus mandibularis*. Although the pathophysiology of tori is not fully understood, it has been shown a relationship between *tori* and a disequilibrium of occlusal forces [43].

Differences between our model and others of the literature (significant loss of weight, 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 because 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.

5. FIGURES AND LEGENDS

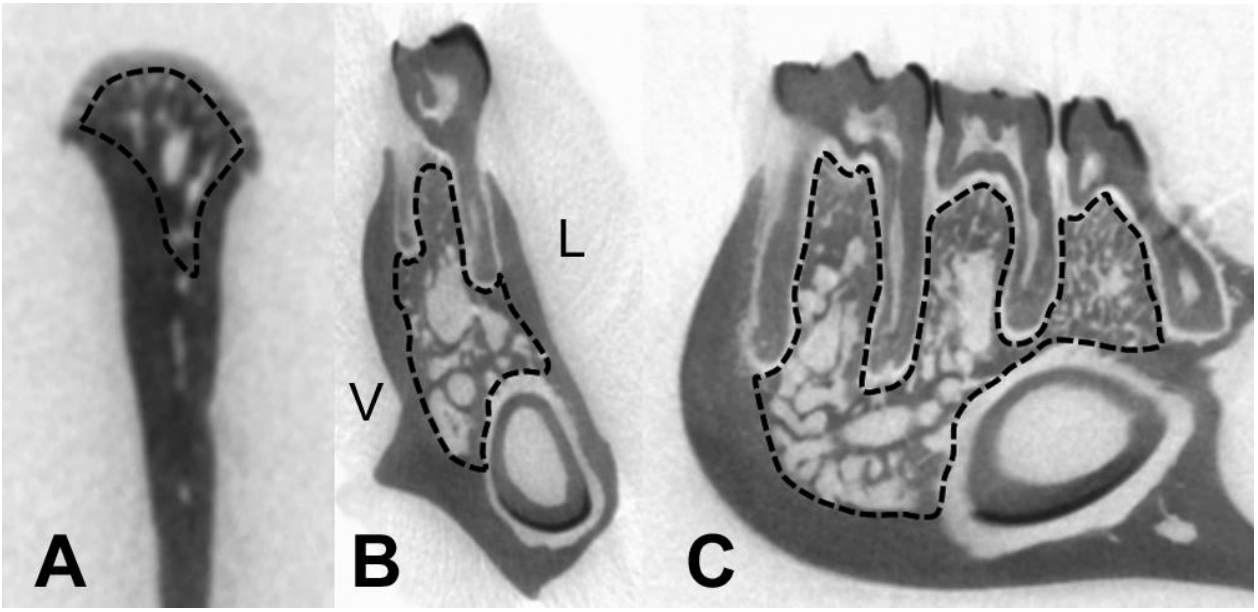

Figure 1. Regions of interest (outlined areas) used to measure BV/TV on 2D sections of the mandible obtained by microCT.

- **A)** Frontal section of a right condylar head (midpoint of the condyles is identified by the presence of medial and lateral poles).
- **B)** Frontal section of a right alveolar region (point of reference: first molar's distal roots). Measuring sites (n=8) of cortical bone thickness are localized on lingual (L) and vestibular (V) sides.
- **C)** Sagittal section of a right alveolar region (point of reference: distal roots of first, second and third molars and mesial root of the first molar).

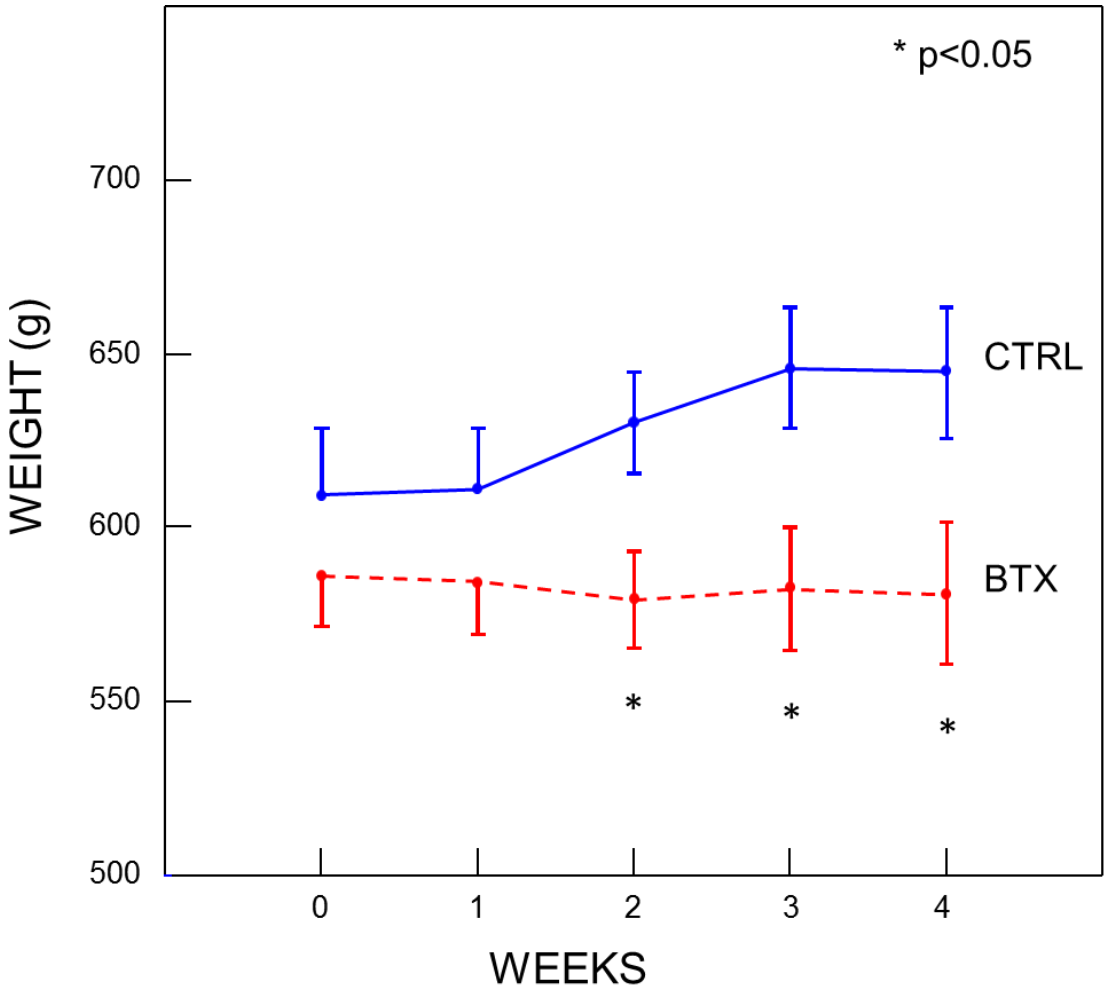

Figure 2. Evolution of the body weight of rats showing significant differences between groups at W 2, 3 and 4.

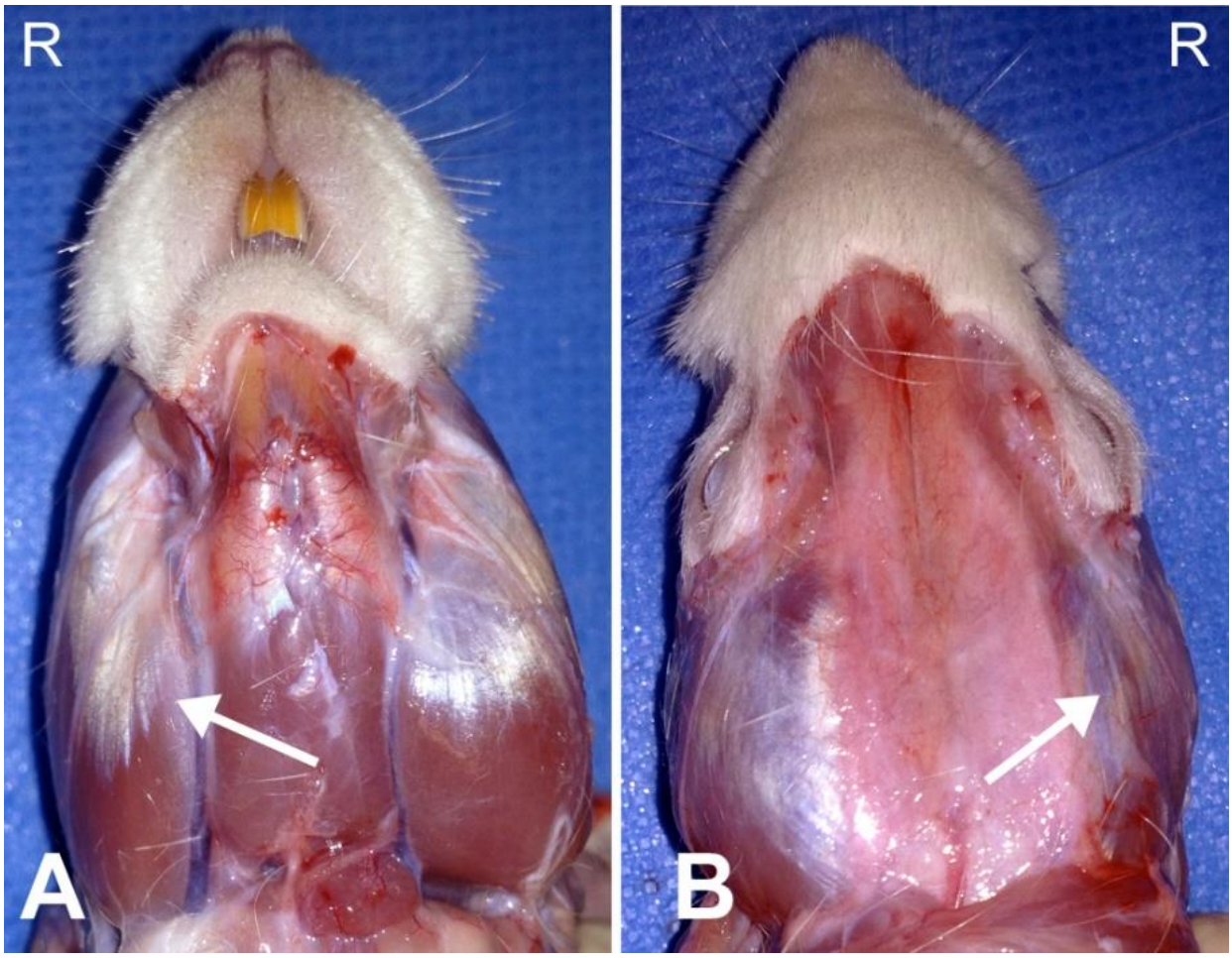

Figure 3. Macroscopic aspect of muscles after BTX injections:

A) Right *Mus Masseter* amyotrophy (bottom view, arrow).

B) Right *Mus Temporalis* amyotrophy (upper view, arrow).

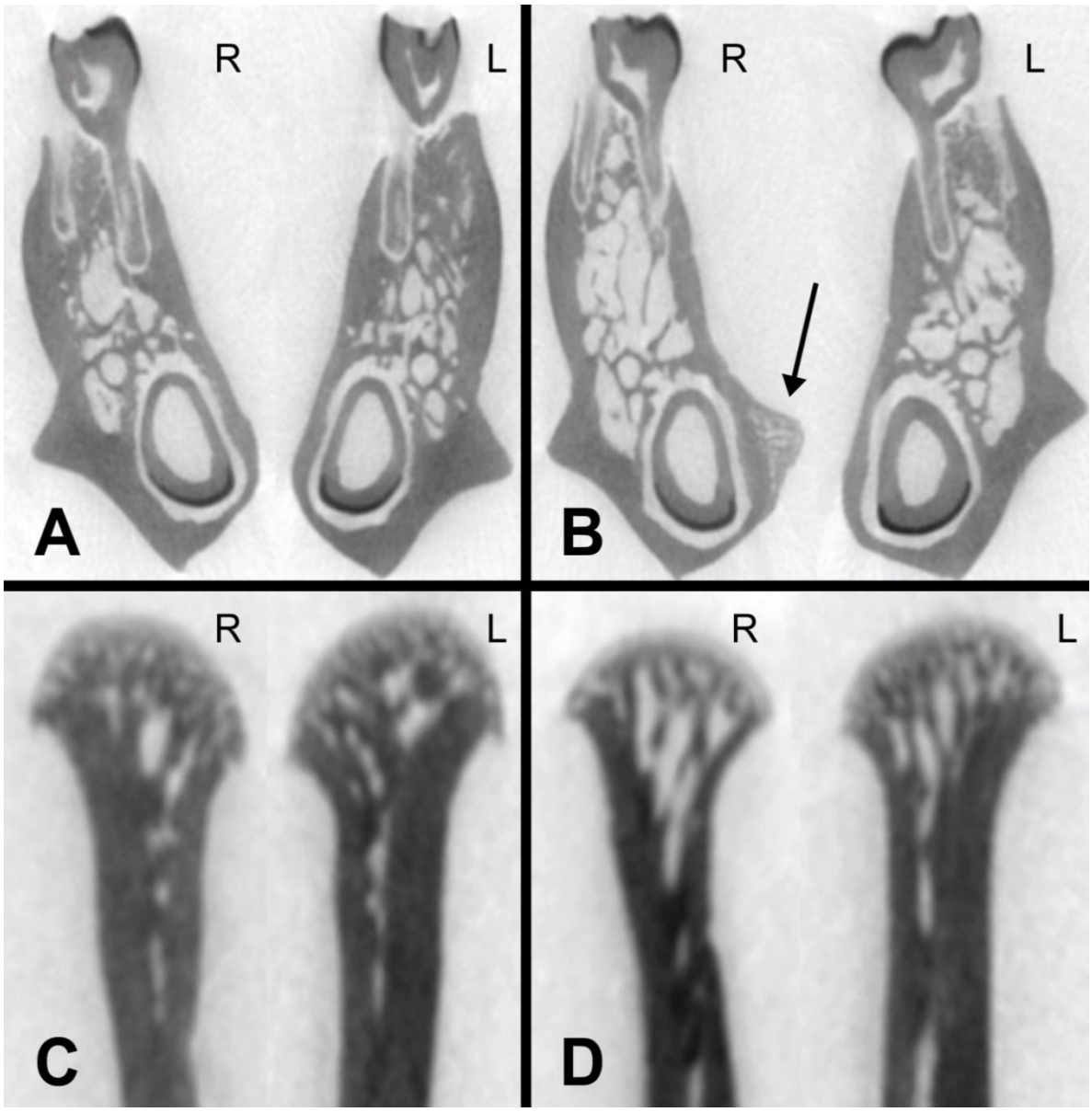

Figure 4. MicroCT analysis of bone loss as seen on 2D sections:

A) Frontal sections of right (R) and left (L) hemimandibles in a rat of the CTRL group showing no difference at the right alveolar bone and at the right *Mus Digastricus* enthesis.

B) Frontal sections of right (R) and left (L) hemimandibles in a rat of the BTX group. Note the alveolar bone loss on the right side and the occurrence of a hypertrophic bone proliferation at the *Mus Digastricus* enthesis (arrow).

C) Frontal sections of the mandibular condylar heads in a rat of the CTRL group showing no difference between each side.

D) Frontal sections of the mandibular condylar heads in a rat of the BTX group showing a marked trabecular bone loss.

For each image R and L identify respectively the right the left side.

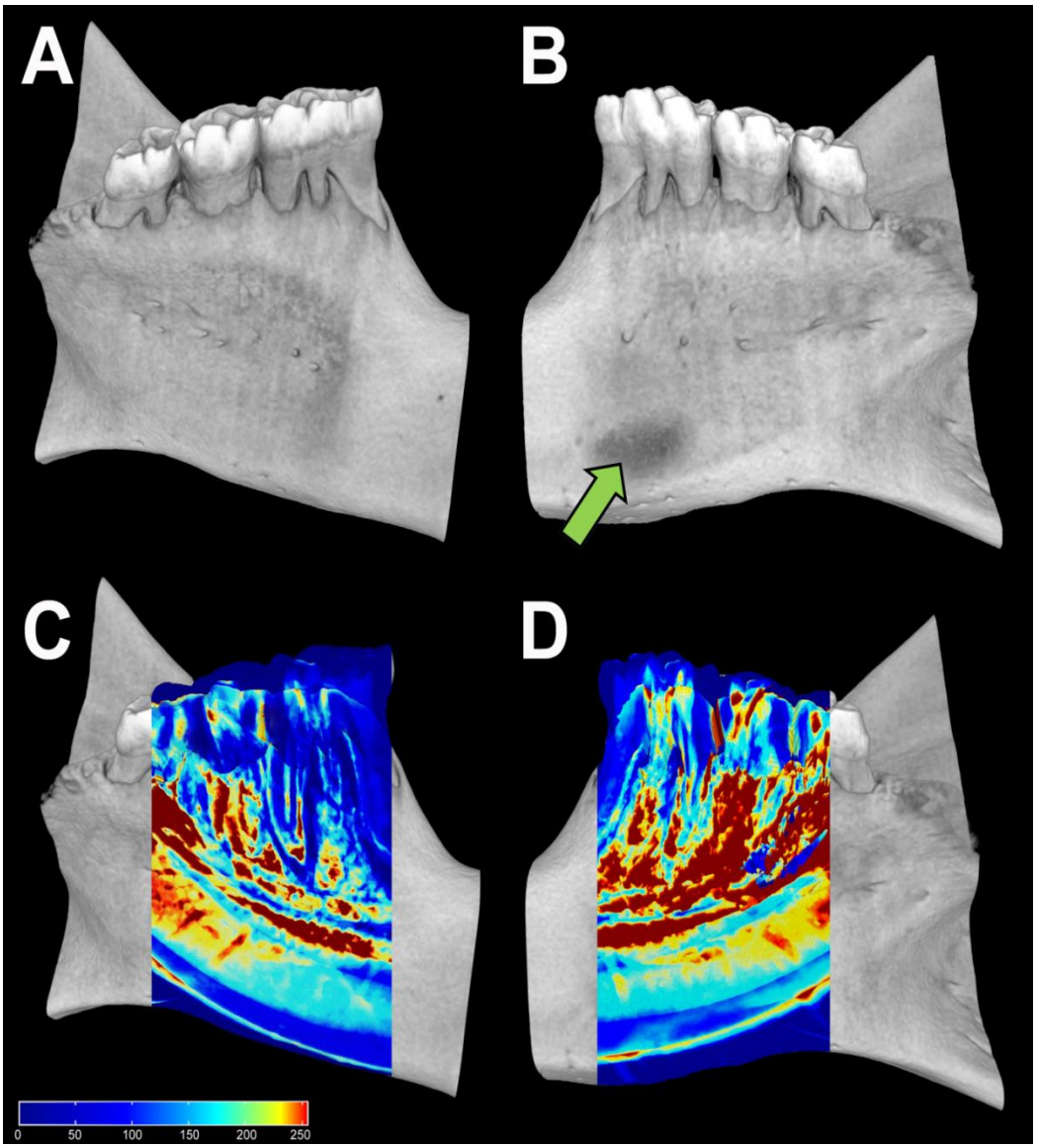

Figure 5. MicroCT analysis of the hemimandibles of rat with BTX paralysis of *Mus Masseter* and *Mus Temporalis* on the right side:

- **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.

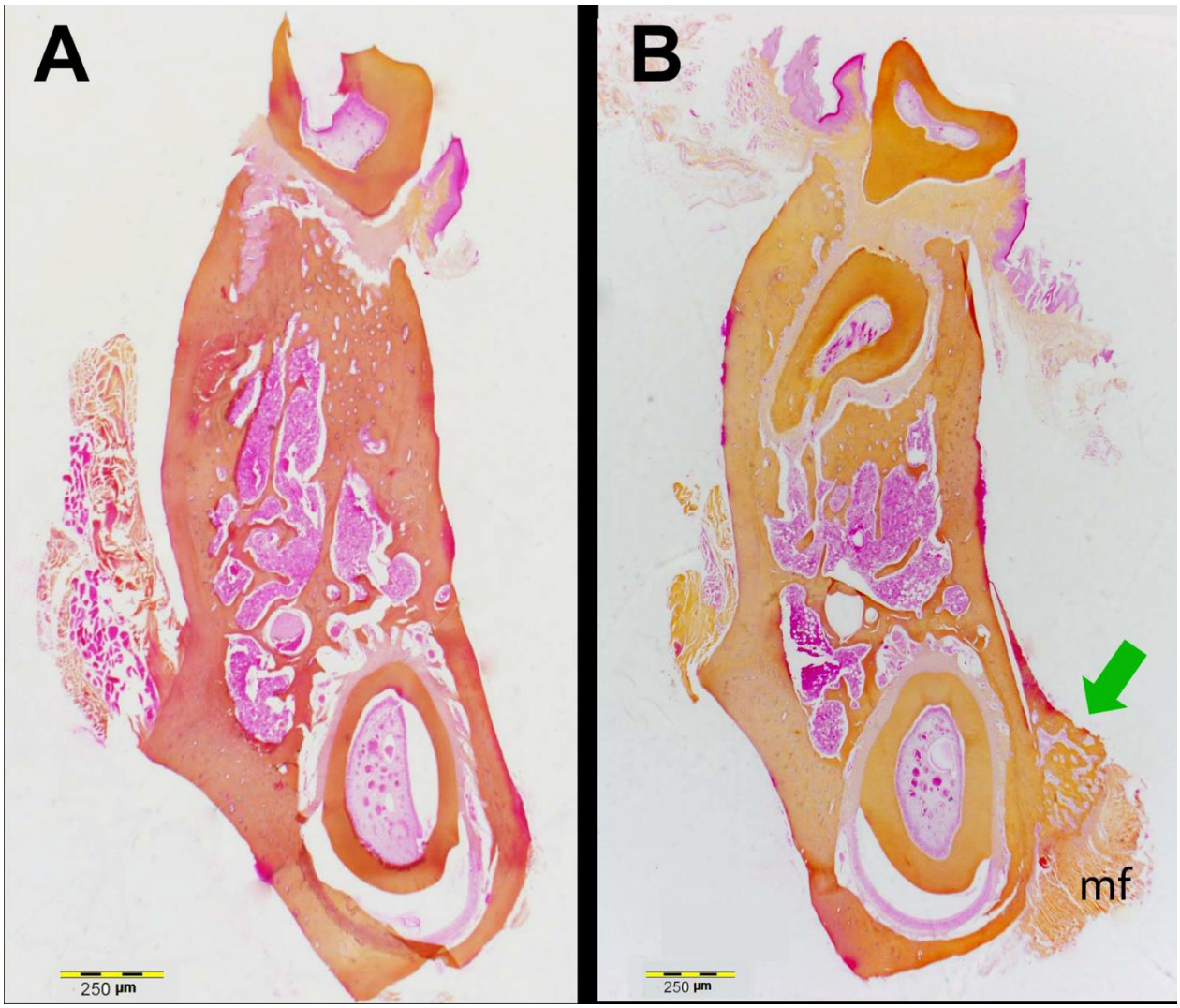

Figure 6. Histological analysis:

A) Frontal section of a right hemimandible from a rat of the CTRL group.

B) Frontal section of a right hemimandible from a rat of the BTX group showing the hypertrophic bone proliferation made of metaplastic bone at the *Mus Digastricus* enthesis (green arrow).

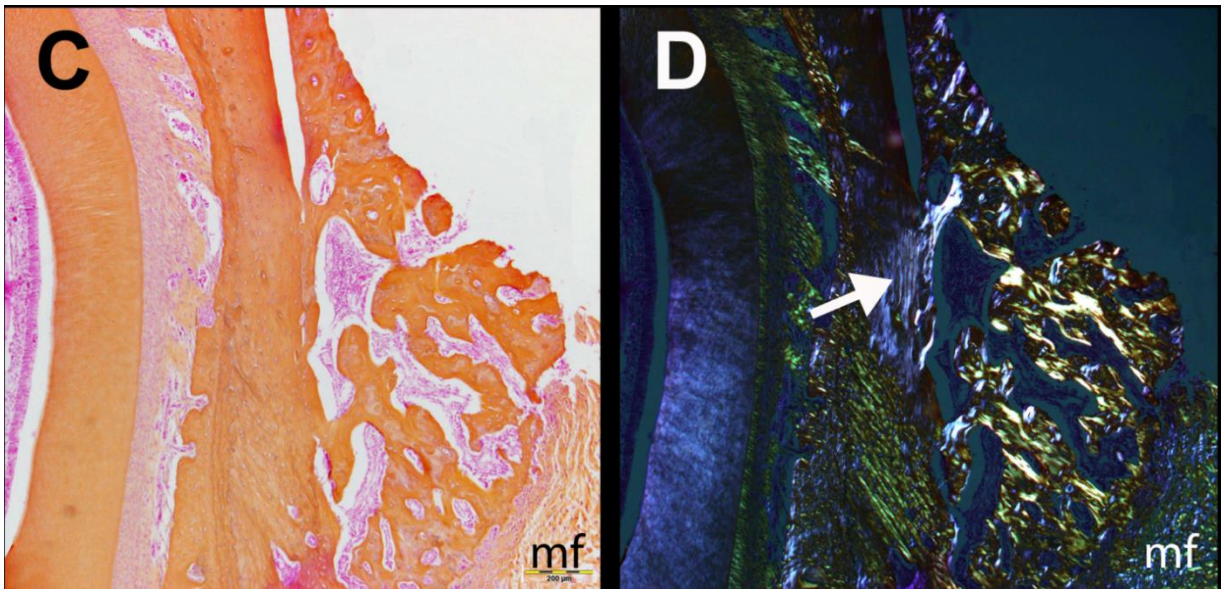

Figure 6. Histological analysis:

C) Higher magnification of the bone proliferation showing: woven bone exhibiting an active remodeling with numerous osteoblast alignments and active osteoclasts. Fibers of *Mus Digastricus* (mf) are anchored at the surface of the bony proliferation

D) Same section seen in polarized microscopy showing normal cortical bone made of lamellar bone (arrow) while the proliferation is composed of woven bone.

Table I: Bone morphometric parameters

	CTRL Left	CTRL Right	BTX Left	BTX Right
Alveolar BV/TV (%)	58.75 ± 1.95	57.99 ± 3.82	61.14 ± 2.91	41.48 ± 2.68 *
Condylar BV/TV (%)	68.18 ± 2.41	63.47 ± 1.65	71.82 ± 1.19	36.49 ± 3.52 *
LCt.Th (µm)	518 ± 14	484 ± 11	519 ± 20	479 ± 22
VCt.Th (µm)	509 ± 18	538 ± 14	506 ± 24	485 ± 20
D_f frontal plane	1.416 ± 0.010	1.420 ± 0.018	1.419 ± 0.009	1.474 ± 0.009 *

The gray boxes indicate a significant difference vs. left side

* indicates a significant difference vs. CTRL

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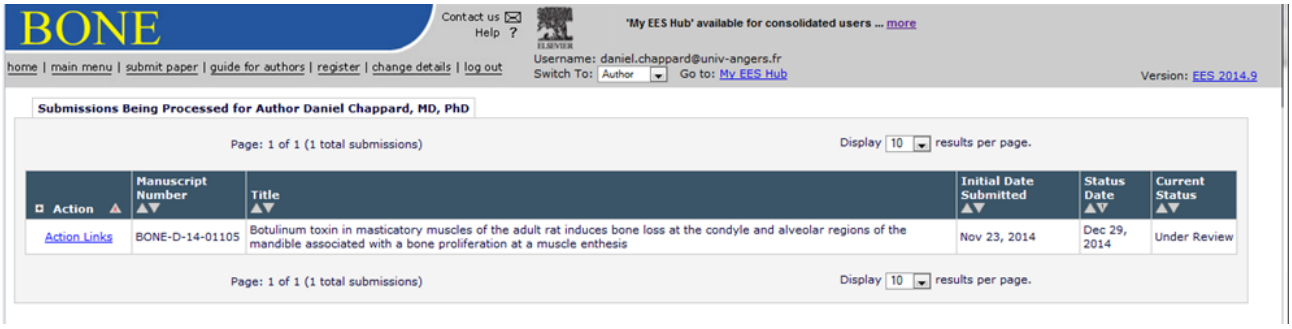
ADDENDUM:

Statut de publication:

Ce travail est soumis pour publication à la revue Bone # BONE-D-14-01105, depuis le 23/11/14 (IF = 4.46).



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