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REVISTA ESTOMATOLOGIA

Original Article

Three-dimensional assessment of enamel and dentine in mouse molar teeth during masseter muscle hypofunction.

Evaluación tridimensional del esmalte y la dentina de dientes molares de ratones durante la hipofunción del músculo masetero.

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SUMMARY

Background: Mouse molar is a widely used model for teeth development. However, the effect of masticatory function on enamel and dentine in adult individuals remains poorly understood. As reported, the unilateral masseter hypofunction induced by botulinum toxin type A (BoNTA) resulted in mandibular bone damage and signs of unilateral chewing in adult mice.
Objective: We aimed to assess the amount of enamel and dentine in the first molar (M_1) during the unilateral masseter hypofunction in mice, using high-resolution X-ray microtomography (µCT) as three-dimensional approach.
Materials and methods: Mandibles of adult BALB/c mice, located either in a Control-group (without intervention) or a BoNTA-group, were ex-vivo scanned using µCT. Treated individuals received each one BoNTA intervention in the right masseter, and saline solution in the left masseter (intra-individual control). Enamel and dentine from M_1 were segmented, and volume, thickness and mesial root length were quantified.
Results: Enamel volume from treated side resulted unchanged after 2 weeks of unilateral masseter hypofunction. No differences for enamel volume were found between both sides of control individuals, and between these and samples from hypofunctional side in BoNTA-group. Enamel volume from saline-injected side was reduced when compared with experimental side ($p<0,01$). No differences in dentine volume, thickness of enamel and dentine, and mesial root length were found for any group.
Conclusion: The amount of enamel in hypofunctional molars remains unaffected after unilateral BoNTA intervention in the masseter, but contralateral side showed reduced enamel volume. Therefore, increased functional wearing during unilateral chewing after BoNTA intervention should be considered.

Key words: Mastication, botulinum toxin type A, X-ray microtomography, unilateral chewing.

RESUMEN

Introducción: El molar de ratón es utilizado como modelo de estudio en el desarrollo dental. El efecto de la función masticatoria sobre el tejido dental en individuos adultos aún se comprende. En ratones adultos, la hipofunción unilateral del masetero inducida por toxina botulínica tipo A (BoNTA) resultó en daño óseo mandibular y signos de masticación unilateral.
Objetivo: Evaluamos la cantidad de esmalte y dentina en el primer molar (M_1) durante la hipofunción unilateral del músculo masetero en ratones mediante análisis con microtomografía (µCT).
Materiales y métodos: Las mandíbulas de ratones BALB/c adultos, del grupo Control (sin intervención) o el grupo BoNTA, fueron escaneadas ex-vivo con µCT. Los individuos tratados se inyectaron con BoNTA en el masetero derecho y con solución salina en el masetero izquierdo (control intra-individuo). El volumen y grosor de esmalte y dentina del M_1, y la longitud de la raíz mesial fueron medidos.
Resultados: No hubo cambios en el volumen del esmalte del lado tratado con BoNTA y en ambos lados del grupo Control, 2 semanas post-intervención. El esmalte

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del lado control intra-individuo se redujo comparado con el lado experimental (p< 0,01). No hubo cambios en el volumen de dentina, el grosor de esmalte y dentina o en longitud de la raíz mesial de ambos grupos. **Conclusión:** La cantidad de esmalte en los molares hipofuncionales no se afecta después de la inyección unilateral de BoNTA en masetero, pero si se reduce en el lado contralateral. Por lo tanto, se debe considerar un desgaste dental asimétrico durante esta intervención.

Palabras clave: Masticación, toxina botulínica tipo A, microtomografía de rayos X, masticación unilateral.

INTRODUCTION

Mastication is a biomechanical process necessary for feeding that involves the complexed and coordinated function of the masticatory muscles (lateral and medial pterygoids, temporalis and masseter) in mammals.[1-3] Each masticatory muscle adds specific vectors and directions to the mandibular movements during the chewing cycle.[2] Disruption of the balanced masticatory function in mature individuals is associated with several pathologic conditions such as tooth loss, parafunctions, severe periodontitis, temporomandibular disorders (TMDs) and unilateral chewing habit.[4-8]

Also, unilateral chewing is considered as a risk factor for tooth surface loss in adult population.[4,8] Moreover, therapeutic interventions such as botulinum toxin type A (BoNTA) injection in the masseter muscle, used for parafunctions, TMDs or aesthetic conditions that may present unilateral signs and/or symptoms.[9-11] result in a decrease of the masticatory function that contributes to the risk of developing a unilateral chewing behavior.[12-14] However, how the masticatory load imbalance induced by BoNTA intervention affects the amount of wear of dental tissues remains poorly understood.

BoNTA is a neurotoxin produced by the anaerobic bacterium, *Clostridium botulinum*, which blocks the release of acetylcholine after intramuscular injection, generating rapid muscle paralysis and subsequent atrophy.[3,15-17] In dentistry, BoNTA has been used as off-label treatment without approved current indications for its use in the masticatory apparatus, and with the masseter muscle frequently targeted.[18,19] The masseter muscle is the biggest and strongest masticatory muscle in mice,[20] and its balanced function is necessary for mandible stabilization during the chewing process.[3] Recently, an in vivo model with adult mice showed bone deleterious effects on mandibular condyle after unilateral BoNTA intervention in the masseter muscle, using high resolution X-ray microtomography (µCT).[21] In fully mature mice, BoNTA injection generates masseter paralysis almost instantaneously, with an established effect after 24 h.[3]

Two weeks after this intervention, the individuals exhibited normal body mass gain, similar to controls without intervention.[17, 21] Therefore, in order to eat the provided food pellets, mice carry out a unilateral chewing pattern that can be evidenced by the differential maxillary incisors wear.[21] Nonetheless, the effect of this condition on the enamel and dentine of mouse molar teeth remains to be investigated.

The availability of genetic manipulation in mice has increased the interest in their use for the study of the pathogenesis of several developmental and degenerative pathologies,[22,23] as well as for the understating of oral motor disorders.[1] In addition, mouse first molar teeth (M_1) are widely used as models for organogenesis and tooth development.[24-28] The mouse M_1 exhibits a special configuration in the occlusal portion, with enamel-free areas and exposed dentin, which is completed due to the functional wearing process at 5 weeks in BALB/c mice, and coincidences with the reach of sexual maturity.[29] Because BoNTA intervention in the masseter muscle decreases the masticatory loading of the injected side, the resulting biomechanical imbalance may increase the functional demand on the contralateral non-paralyzed side.[13] Therefore, the aim of this study is to assess the hypothesis that, during the unilateral masseter hypofunction induced by BoNTA in adult mice, the molars are underused and that therefore the enamel volume of the contralateral (non-affected) side is reduced in the M_1 compared to the hypofunctional side.

MATERIALS AND METHODS

Animal model

All procedures were approved by the CICUA (Institutional Animal Care and Use Committee) of Universidad de Chile under certificate Nº 17011-OD-UCH. Eight adult BALB/c mice (9 weeks-old) were injected once with 0,2 U BoNTA in 10 µl (Onabotulinumtoxin A; BOTOX®, Allergan Chile; Lot #C4306C2) in the right masseter, and the same volume of BoNTA vehicle (10 µl; saline solution; 0,9% w/v NaCl) was injected in the left masseter, used as intra-individual control (BoNTA-group). This procedure was performed under general anesthesia using a combination of Ketamine/Xylacine (80 mg/kg and 8 mg/kg, respectively). Nine animals of the same age and gender, without any intervention, were used as baseline samples (Control-group). All animals were housed during 2 weeks with water and food ad libitum, under standard and controlled conditions in the animal facility of the Faculty of Dentistry (Universidad de Chile). All animals were provided with the same standard cylinder-shaped feed pellet (LabDiet® JL Rat and Mouse/Auto 6F 5K67; LabDiet, USA). At the end of the experiment, all mice were euthanized using an intraperitoneal injection of anaesthetic overdose. Then, mandibles were obtained and stored at room temperature in formalin solution (10%, neutral buffered, Sigma Aldrich®, USA) prior to µCT scanning procedures. All animal samples were acquired from a previously published study.[21]

µCT scanning

Mouse mandibles were scanned using ca-

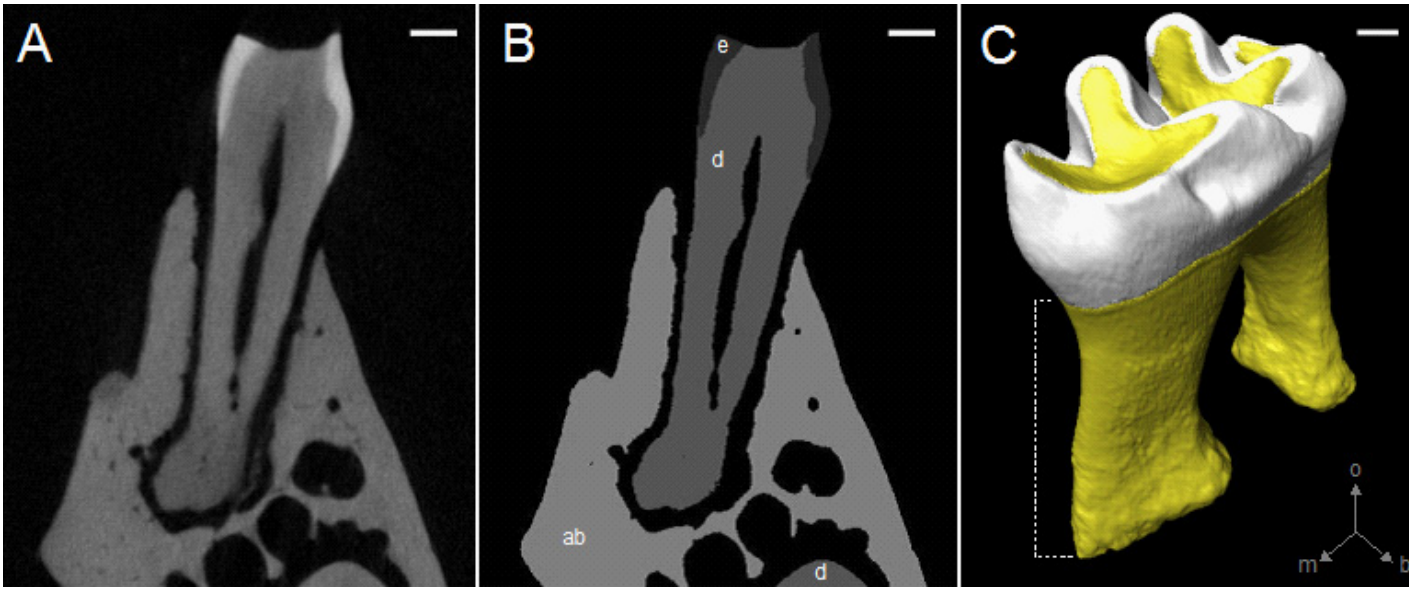

Figure 1. High resolution µCT data processing. (A) Representative raw µCT 2D image at mesial root level of M_1 from saline-injected side (left) of an individual from BoNTA-group, with a voxel size of 5.1 µm. (B) Same µCT 2D image after semi-automatic segmentation showing three separated materials (enamel, dentine and bone). (C) Bucco-lingual view of 3D reconstructed left M_1 showing enamel (white) and dentine (yellow) without the surrounding alveolar bone tissue. Dashed square bracket illustrates the mesial root length measurement between enamel-dentine junction (mesial side) and the most apical portion of the root. Abbreviations: e, enamel; d, dentine; ab, alveolar bone; m, mesial; o, occlusal; b, buccal. Scale bar: 200 µm.

librated parameters (voxel size: 5,11 µm; voltage: 80 kV; current: 60 mA; integration time: 1500 ms; 0,5 mm aluminium filter) on a DIONDO d3 microCT equipment (DIONDO GmbH, Germany). In Avizo 9,2 (Thermo Scientific™, USA), the obtained 2D images were used to segment the whole 3D volume around the crown and roots of the first mandibular molar (M_1) (Figure 1A). A threshold-based selection with a manual correction was implemented to select specifically the enamel and dentine. Subsequently, 3D stacks were exported and processed semi-automatically with a diffusion algorithm (BIOMEDISA, Biomedical Image Segmentation App)[30] in order to segment three materials: enamel, dentine and bone (Figure 1B).

Teeth analysis

The resulting segmented files were reconstructed in 3D in Avizo, and the volume statistics were obtained for enamel and dentine materials, separately (Figure 1C). Also, to determine mesial root length, linear measurements were obtained between enamel-dentine junction in the middle mesial side and the most apical point of the mesial root (Figure 1C). Each material (enamel and dentine) was then exported as a 2D-stack to be converted in 8-bit images using ImageJ 1,52e.[31] Afterwards, enamel thickness and dentine thickness were quantified following the protocol for Trabecular Thickness (TbTh) in the BoneJ extension.[32] All measurements were performed by a single blinded observer. 3D depictions of the output for enamel and dentine thickness from BoneJ were developed using Paraview 5.4.1.[33]

Statistical analysis

The results are reported as Min to Max or mean ± standard deviation (s.d.). Differences between experimental and control sides were assessed using paired *t*-test (intra-individual) or unpaired *t*-test (between individuals). Normality distribution of the data was determined with the Shapiro-Wilk test. All statistics were performed in GraphPad Prism 8.0.1 (GraphPad Software, La Jolla, CA, USA). Statistical difference was chosen for a value of $p<0,05$.

RESULTS

Enamel volume and mesial root length of the M_1 do not change during unilateral masseter hypofunction.

In the BoNTA-group, total enamel volume of the M_1 from the hypofunctional side remained unchanged after 2 weeks (0,159±0,005 mm^3), when compared with samples from Control-group (0,158±0,004 mm^3). In contrast, enamel volume in the samples from the saline-injected side was significantly reduced (0,151±0,006 mm^3) (Figure 2). There was no significant difference in total enamel volume between the left and right sides of the Control-group and hypofunctional side of BoNTA-group (Figure 2). Unlike for enamel, we did not

find any difference in the total dentine volume between sides in BoNTA-group and Control-group (Figure 2).

A qualitative evaluation using 3D depictions of enamel and dentine thickness was performed, and no differences were found in the overall pattern of thickness distribution in the M_1 from both sides in samples from BoNTA-group and Control-group (Figure 3). Only minor changes consistent with increased wearing were observed in the lingual cusps of the M_1 from the saline-injected side (Figure 3). However, quantitative assessment of enamel and dentine thickness revealed no significant changes after 2 weeks of unilateral masseter hypofunction for any group (Table 1). Linear measurements of mesial root length also demonstrated no changes in treated and control individuals (Table 1).

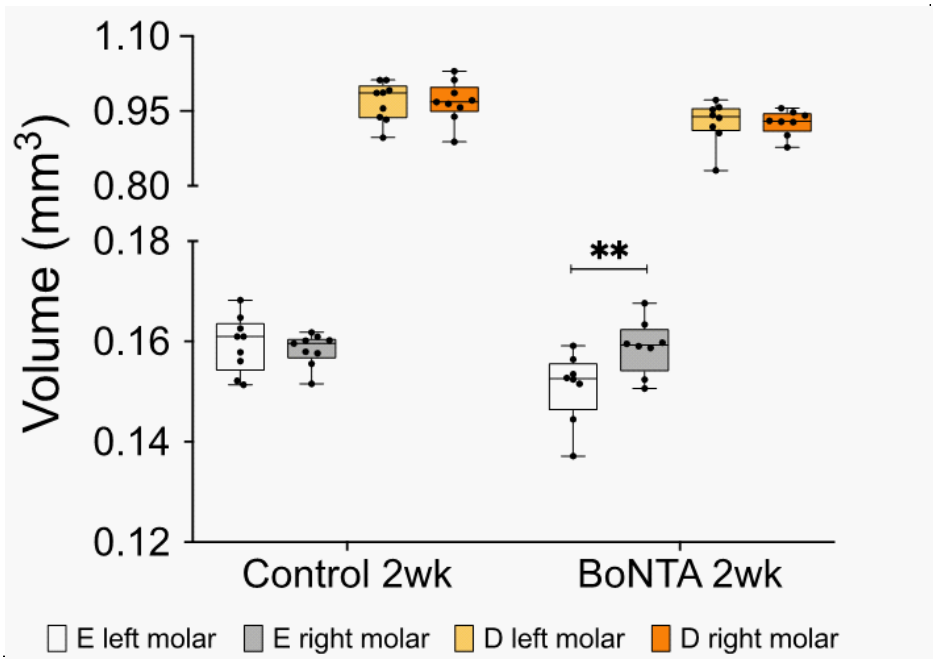

Figure 2. No enamel volume loss in hypofunctional molars. BoNTA intervention is followed by rapid masseter muscle paralysis and, after 2 weeks, the enamel volume of the M_1 from the sa-line injected side (intraindividual control) is reduced when compared with the same tooth from the BoNTA-injected side. No difference was found in the enamel volume between sides in the samples from Control-group, and between these and M_1 from hypofunctional side (unpaired *t*-test; p>0,05). In addition, no changes in dentine volume was determined in both groups. N = 8-9; Min to Max; Shapiro Wilk test, p>0,05; paired *t*-test; **: p<0,01. Abbreviations: BoNTA, botulinum toxin type A; E, enamel; D, dentine; wk, weeks.

DISCUSSION

Our results demonstrate that, during the unilateral masseter hypofunction induced by BoNTA in adult mice, the enamel volume of underloaded M_1 remains unchanged after 2 weeks, and the enamel volume from the control side (non-affected) samples was reduced. In addition, no changes in the dentine volume were found for any group.

These findings were obtained with a µCT approach, which is a non-invasive and a non-destructive technique. When combined with our mouse model, this technique represents a powerful tool to assess the dental mineralized tissues in 3D without destroy them, which is advantage compared with other techniques (i.e. a histological approach). Also, the use of µCT data allows to test several hypotheses related with very different tissues (i.e. dental versus bone and cartilage tissues in the mandible) without the unnecessary use of additional animals for each experiment. Moreover, three-dimensional evaluation of enamel with µCT is also possible in vivo, using specific equipment, allowing to the follow-up of dental abrasion under designed conditions using the same individual.

The morphology of mouse molar teeth exhibits similarities with human teeth.[34] Because of the easy handling, low-cost maintenance, easy breeding and advanced genetic manipulation, mice have become an essential model for the study of developmental and acquired disorders of craniofacial structures, like teeth, in mammals.[28,34] Also, the characterization of the enamel on a widely used strain, the BALB/c, allows us to understand the effect of altered masticatory function in these adult individuals.[29] During the postnatal period, after eruption, mouse M_1 presents immature and aprismatic enamel covering the tip of the cuspids until 5 weeks of age, when this feature is lost due to normal abrasion.[29]

The three-dimensional arrangement of the molar enamel in mice is well described, and substantial differences between these teeth and the incisors should be considered during experimental settings.[24] The human molar teeth exhibits covering enamel in the occlusal surface, which is thicker in the lateral portions of the cuspids than in the cusps tips.[35] Unlike humans, C-type enamel configuration (schmelzmuster) of mice presents enamel-free occlusal areas.[24,36] Thus, excessive functional wearing of the occlusal enamel may affect average enamel thickness during three-dimensional evaluation in humans, but the absence of occlusal enamel in mouse M_1 may explain why this measurement did not change during unilateral masseter hypofunction.

However, there was a significant decrease in the enamel volume in the samples from contralateral (non-affected) side in the individuals of BoNTA-group, which is

consistent with increased dental abrasion on that side. This difference in the enamel volume between sides was not found in the molars from Control-group, and between these measurements and those obtained from M_1 of BoNTA-injected side in experimental group, demonstrating the influence of balanced masticatory muscle function on the amount of enamel in molar teeth. Here we do not test the activity of masticatory muscles, but results from other studies show a reduction of the EMG activity and a decrease of the mechanical loading on the mandible after BoNTA intervention in the masseter muscle.[13] This outcome suggests that, during unilateral masseter hypofunction, an increase in the activity of the saline-injected side compensates the intake of food (assessed by following the body mass) and generates an increase in dental abrasion. A study that assessed the effect of occlusal hypofunction on mouse mandibular M_1, after reducing the cuspids of the maxillary first molar, an elongation of the mesial root and the alveolar bone around M_1 was reported after 2 weeks in adult mice (9 weeks-old).[37] In contrast, using mice of similar age, we did not find any changes in the mesial root length of the M_1 from the BoNTA-injected side, after 2 weeks of unilateral masseter hypofunction. In addition, no changes in the dentine volume and thickness were found in samples from BoNTA and control sides.

These differences are related to the fact that, unilateral masseter hypofunction, when induced by BoNTA, alters the masticatory balanced but does not eliminate the occlusal contact between molar teeth, in contrast to the mouse model of occlusal hypofunction.[37] Interestingly, consistent qualitative changes were detected in the enamel thickness of the lingual cusps of the M_1 from saline-injected side, when compared with teeth from hypofunctional side (Figure 2). These changes suggest local dental wear that may be related to the path of the mandible during chewing when unilateral masseter hypofunction is induced. This is consistent with the lateral mandibular movements previously descri-

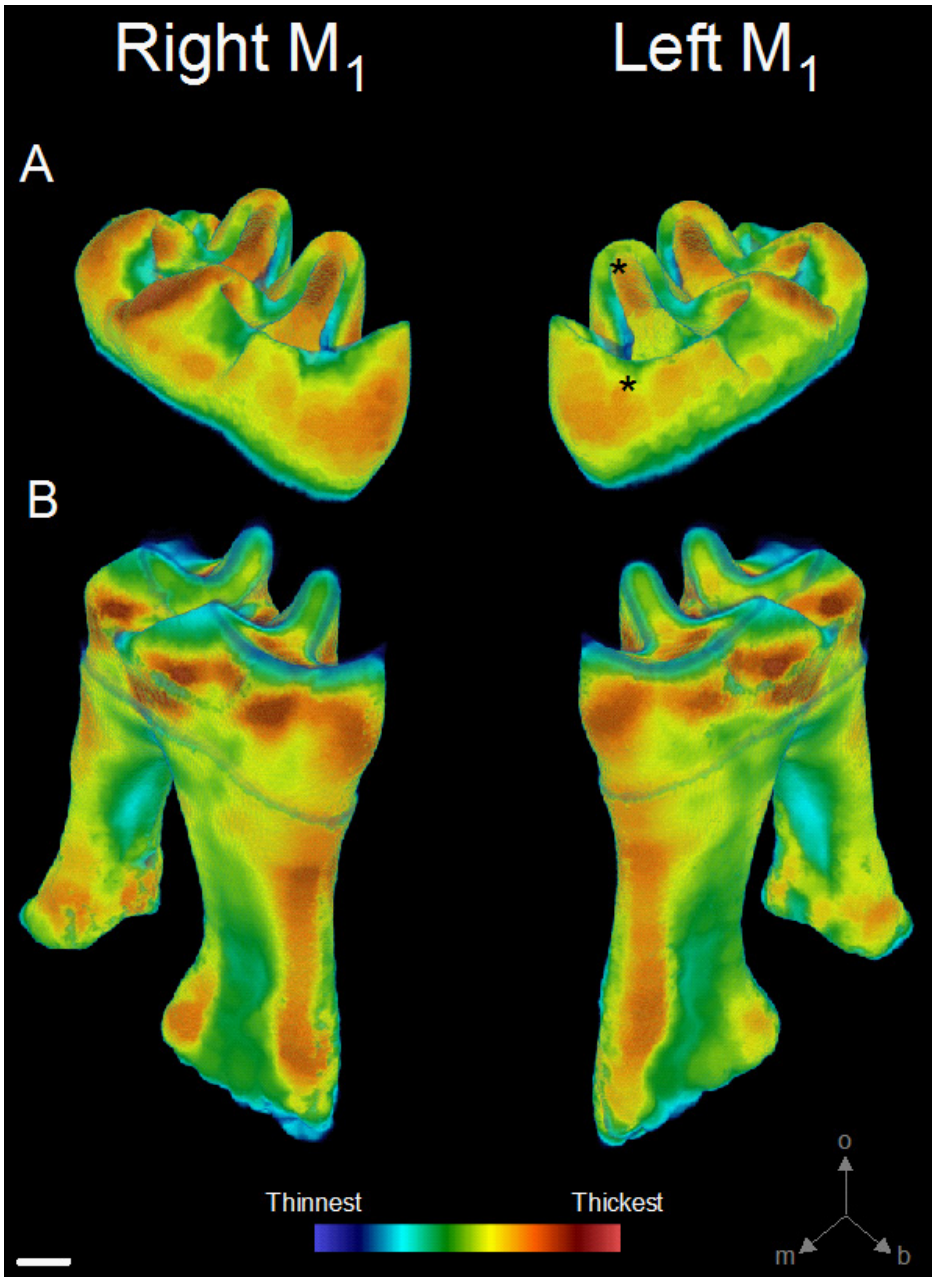

Figure 3. Overall enamel and dentine thickness of M_1 do not change during unilateral masseter hypofunction. Representative 3D depictions of the M_1 from BoNTA-injected side (Right M_1) and saline-injected side (Left M_1) from the same individual previously showed in Figure 1. (A) Mesio-buccal view of enamel thickness shows similar pattern distribution in M_1 from both sides, with only minor qualitative differences in lingual cusps (black asterisks): the cusp tip of L2 and the mesial marginal ridge of L1 (according to the nomenclature proposed by Lyngstadaas et al 1998) (B) Mesio-buccal view of dentine thickness 3D distribution in M_1, with no regional variations between sides. Similar results for both enamel and dentine thickness were found in left and right samples from Control-group. Color scale in gray values. Scale bar: 200 µm. Abbreviations: M_1, irst molar; m, mesial; o, occlusal; b, buccal.

bed in mice, where the masseter muscle function is required.[3] Therefore, masseter muscle from non-affected side may add vectors for anterior and lateral mandibular excursions associated with these local differences in enamel thickness, as result of unilateral chewing behavior.

Here we used the three-dimensional evaluation to assess changes in the enamel and dentine of M_1 in a mouse model. A study showed that the three-dimensional assessment of the mouse M_1 is possible using reconstructions from histological slides during its development.[25] However, histological techniques are not conservative with samples. On the other hand, µCT analysis is a recognized imaging technique to the study of teeth without the destruction of the samples,[38] which supports the idea that efforts in acquiring this kind of technology for research purposes in Colombia (and South America, in general) should be considered. In addition, segmentation of materials from µCT data here exposed can be further developed in order to determine the specific changes of enamel and dentine in human populations during different conditions where unbalanced masticatory function can be found, such as bruxism, severe periodontitis, TMDs, prosthetic rehabilitation and tooth loss.[5,6,8-11]

Importantly, since unilateral chewing is associated with increased tooth wear in adult population, this methodology could take advantage of diagnostic imaging to understand the side effects of unilateral BoNTA intervention beyond the injected muscles, in this case, teeth structure. Also, the study of enamel microstructure using a µCT approach is a reproducible technique that can be complementary to current methods for the evaluation of genetically modified mice,[28] which can improve our understanding of developmental disorders of teeth and associated structures.

CONCLUSIONS

Our results support the hypothesis that enamel volume, during the unilateral masseter hypofunction induced by BoNTA in adult BALB/C mice, is not affected in the M_1 from hypofunctional side, when compared with the enamel volume of molars from the contralateral (non-affected), which is reduced due to increased functional wearing. Moreover, we showed that the use of µCT data, a non-destructive technique, supports the 3Rs statements by reducing the use of animals through the implementation of the same imaging data to test several hypotheses, and by refining the methods with reliable quantitative outcomes. Also, our findings suggest that, after unilateral BoNTA intervention, asymmetry of teeth structure may be expected and should be evaluated periodically in order to avoid undesired side effects. It is also important to highlight that this model of unilateral masseter hypofunction may improve our understanding about the effect of the masticatory muscle function imbalance on dental enamel, which is related with highly prevalent conditions such as severe periodontitis, unilateral chewing habits, as well as with dental prosthetic interventions and aesthetic procedures.

Table 1. Enamel thickness, dentine thickness and mesial root length of M_1

Characteristic	Control-group		BoNTA-group	
	Left M_1 N=9	Right M_1 N=9	Left M_1 N=8	Right M_1 N=8
Enamel thickness (µm)	63,7±1,26	63,1±0,92	62,4±0,95	62,7±1,17
Dentine thickness (µm)	211±3,23	210±3,74	206±6,38	207±4,08
Mesial root length (mm)	1,91±0,07	1.92±0,05	1,90±0,04	1,87±0,07

Results showed as mean ± s.d. Shapiro Wilk test, $p > 0.05$. Paired *t*-test between sides of the same group. Unpaired *t*-test between samples from different groups. Abbreviations: BoNTA, botulinum toxin type A; M_1, first molar.

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CONFLICT OF INTEREST AND SOURCE OF FUNDING

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