Original

Micro/nanostructural Characteristic Changes in the Mandibles of Rats after Injection of Botulinum Neurotoxin

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Abstract: Our objective was to perform a quantitative evaluation of changes in the micro/nanostructural characteristics of entheses in rats with reduced masticatory muscle functional pressure, with the aim of elucidating the mechanism whereby masticatory muscle functional pressure contributes to growth and development of the mandible from a biomechanical perspective. Male Wister rats aged 4, 11, 18 and 25 weeks were divided into a Botox group injected with a botulinum toxin serotype A formulation to reduce muscle function (BTX) and a control group (CTRL). They were euthanized 6 weeks later and bone quality at the masseter insertion on the mandibular was analyzed. In the BTX group, the number of fibrous chondrocytes at entheses was significantly lower than in the CTRL group at all ages. The diameter of collagen fiber bundles in rats in the BTX group injected with BoNT/A during their growth phase was significantly smaller than that of rats in the CTRL group. In the mandibles of rats in the CTRL group the preferential alignment was consistent with the orientations of the muscle and tendon, but in growing rats treated with BTX, it was less closely aligned with the orientations of the muscle and tendon.

Key words: Bone quality, Collagen fiber, Biological apatite crystallite, Microbeam X-ray diffraction, Second harmonic generation imaging

Introduction

Humans normally start suckling by infant swallowing immediately after birth, and gradually achieve adult swallowing by around the age of 5–6 months as the primitive reflex is lost in the process of cerebral development1. During the suckling period, they primarily ingest food using the facial muscles, but once the teeth erupt they start using the masticatory muscles to eat. The acquisition of masticatory muscle function is therefore believed to play a major role in the growth and development of the maxillofacial region, particularly the lower face, including the lower part of the face below the nasal floor is closely associated with the growth of the floor of the braincase, the growth of the suture regions, and the development of respiratory function, whereas the growth and development of the lower part of the face below the nasal floor is closely associated with masticatory muscle-dependent jaw function.2-3

Frongia et al. investigated masticatory muscle function in 17 patients with jaw deformities, and reported that masticatory muscle function was bilaterally unbalanced.4

Myofunctional therapy has been shown to eliminate the functional pressure of the masticatory muscles from the mechanical environment in the maxillofacial region, promoting the healthy development not only of the dentition but also of the maxilla and mandible, and is now widely used.5-10 Inoue et al. found that osteocytes were activated in mice given a high masticatory load, generating new bone at entheses of the masticatory muscles, and therefore these muscles undoubtedly have a major effect on the bone dynamics of the mandible.11

Functional pressure, which is believed to contribute to maintaining the homeostasis of the mandible, is highly diverse, and much remains unclear concerning mechanical environment that regulates the homeostasis of the mandible. This is a special bone that is particularly susceptible to changes in functional pressure, which not only brings about changes in its internal structure but also affects its external shape.12-15 It is thus difficult to perform a quantitative analysis-based assessment even of the association between the external forces acting on the jawbone and the mechanical stress generated within the bone.

As the first reports that the mechanical environment of bone is strongly reflected in its micro/nanostructural characteristics, studies...
have been conducted regarding the accurate prediction of the mechanical function of bone by bone quality analysis16-18).

Some of these studies have demonstrated that collagen fibers and biological apatite (BAp) crystals, the main components of bone extracellular matrix, are both influential factors in bone quality that resist the tensile and compressive stresses imposed on bone tissue19-20). Vashishth et al. reported that age-related changes in collagen bridge structure are a major cause of reduced bone strength21). Warshaw et al. discussed the association between the mechanical environment and collagen fiber orientations within bone, and showed that they are an influential factor in bone quality that reflects the direction of load on the bone22). BAp crystals are ionic crystals with a highly anisotropic hexagonal crystal nanostructure, and the crystal c-axis is predominantly aligned with the load direction22). Nakano et al. used microbeam X-ray diffractometry for the quantitative analysis of the alignment of BAp crystals in the trunk and limb bones of experimental animals, and identified a strong correlation between bone mechanical function and BAp crystal alignment23-25). Because bone quality factors such as bone structure, bone matrix, calcification degree, microdamage, and bone turnover strongly reflect the local load environment, a quantitative analysis of the histological morphology of bone at entheses of the masticatory muscles and its micro/nanostructural anisotropy should make it possible to predict the mechanical function of the mandible with high accuracy.

Our objective in this study was therefore to carry out a quantitative evaluation of changes in the micro/nanostructural characteristics of entheses in rats with reduced masticatory muscle functional pressure, with the aim of elucidating one aspect of the mechanism whereby masticatory muscle functional pressure contributes to the growth and development of the mandible from a biomechanical perspective.

Materials and Methods

Animal experiments were approved by the Ethics Committee of Tokyo Dental College (Ethics Application Number: 302801). Every possible effort was made to minimize animal suffering.

Experimental animals

Male Wistar rats aged 4, 11, 18, and 25 weeks (each n = 20) were divided into a Botox group injected with botulinum toxin serotype A (BoNT/A) formulation (Botox Vista®, Allergan, Irvine, CA, USA) to reduce muscle function (BTX, n = 10) and a control group (CTRL, n = 10). When deciding the ages to use, rats aged 4 and 11 weeks were used as growing rats and those aged 18 and 25 weeks as mature rats following Hamada et al.’s study showing that the rat mandible continues to grow until approximately 14 weeks of age, when it approaches its mature shape26). Experimental animals were kept in plastic cages with free access to drinking water and solid chow. Body weight measurement was n = 10 and other measurements were n = 5.

Surgical preparation

Experimental animals were anesthetized with sevoflurane. They were stabilized in a supine position after anesthesia with intraperitoneal injection of a mixture of 0.15 mg/kg body weight medetomidine, 2.0 mg/kg body weight midazolam, and 2.5 mg/kg body weight butorphanol. Rats in the BTX group were injected in the right masseter in the angle of the mandible with 5.0 U (0.3 ml) of BoNT/A formulation diluted in physiological saline (Fig. 1A)27,28). Rats in the CTRL group were injected in the right masseter with physiological saline (0.3 ml). They were then kept for 6 weeks before being placed under deep anesthesia with sevoflurane and euthanized (Fig. 1B).

Weight measurements

Rats were weighed before euthanasia. After euthanasia, the masseter was harvested from the origin of the zygomatic arch and the end of the muscle on the mandible, and its weight was measured.

Designation of region of interest

The basic axes of the specimens were assigned as follows: the mesial-distal direction as the X-axis, the direction perpendicular to the mandibular plane as the Y-axis, and the direction perpendicular to the Y-axis as the Z-axis (Fig. 2A)29). The region of interest was designated as the masseter insertion in the mandibular third molar region (Fig. 2B).

Tissue slice preparation

To prepare specimens for histological investigation, they were fixed in 4% paraformaldehyde phosphate buffer solution, and decalcified in 10% ethylenediaminetetraacetic acid for 4 weeks. They were then embedded in paraffin by the usual method, and sliced about 5 µm thick in the YZ plane (frontal sections). These were then stained by Masson’s trichrome method, and calculated the numbers of fibrous chondrocytes in areas on the tendon-bone margin approximately 50 micrometers in all directions from the lateral edge of the masseter insertion in the mandible.

Weighed masseters were frozen immediately and mounted on a cork block. Cryostat sections of 4 µm thickness were prepared at right angles to the longitudinal axis of the muscle fibers. The region of interest was designated at the central point between the origin and the insertion of the masseter. After hematoxylin and eosin staining, the mean cross-sectional area of each masseter muscle fiber in an approximately 0.5-mm-square area was calculated. At the same time, specimens were embedded in autopolymerizing acrylic resin for the preparation of polished sam-
Sagittal cross-sections of these specimens were prepared using a saw microtome with a 300 μm blade (SP1600; Leica Microsystems Inc, Wetzlar, Germany), and these were then sanded using waterproof abrasive paper (Kovax Corporation, Tokyo, Japan) of increasing grit (400, 800, and 1,200) to prepare thin, 200 μm slices.

**Second harmonic generation imaging**

Second harmonic generation (SHG) images were acquired using a multiphoton confocal microscopy system (LSM880 NLO, Carl Zeiss AG, Oberkochen, Germany) with an excitation laser (Chameleon Vision II, wavelengths: 680-1080 nm; repetition rate: 80 MHz; pulse width: 140 fs; Coherent Inc., Santa Clara, CA, USA ) and an objective lens (Plan-Apochromat 20x/0.8 M27; Carl Zeiss AG, Oberkochen, Germany). The excitation wavelength for observation of collagen fibers was 880 nm. Image acquisition, processing for orthogonal views and cropping were performed using ZEN Black (Carl Zeiss). After image acquisition, collagen fiber bundles were quantitatively assessed using an Imaris 8.4 (Bitplane AG, Zürich, Switzerland).

The assessment method comprised tracing the collagen in collagen fiber bundles within bone approximately 150 micrometers from the ridge of the masseter in the mandible in all directions, and calculating their mean diameter. The angles of collagen fiber bundles in the tendon within the images were also measured.

**BAp crystal alignment**

An optical curved imaging plate (IP) X-ray diffractometer (XRD: D/MAX PAPIDII-CMF, Rigaku Corporation, Tokyo, Japan) was used for the quantitative evaluation of BAp crystal alignment. Measurements were made by two methods; those with the transmission optical system and the reflecting optical system, both of which used Cu-Kα radiation as the beam source. The tube voltage was 40 kV and the tube current was 30 mA. The irradiation field was determined using the optical microscope attached to the XRD (×0.6-4.8 magnification), and the incident beam was a circular microbeam of diameter 100 μm. The reflecting optical system was used for measurements in the X-axis direction and the transmission optical system for measurements in the Y- and Z-axis directions, and the diffraction X-ray beam was detected using a curved IP.

Analysis conditions followed the method used by Nakano et al. Using 2D Data Processing software (Rigaku, Japan), the X-ray intensity ratios of the two diffraction peaks in the 002 and 310 planes were calculated from the diffraction ring images. Increase in the intensity ratio indicated an increase in c-axis alignment of the BAp crystallites.

**Statistical analysis**

In statistical analysis, mean values were calculated and an unpaired t test was used for comparisons between two groups of rats of the same age. A value of $p < 0.05$ was regarded as statistically significant.

**Results**

**Body weight and Masseter muscle weight**

There were no significant differences between the body weights of rats in the BTX and CTRL groups (Fig. 3). Masseter muscle weights
Figure 4. Masseter muscle weight measurements. The masseter muscle weighed less in the BTX group. Data indicate means ± SEM, n=5 independent experiments; *p<0.05, **p<0.01, ***p<0.001 compared to CTRL.

Figure 5. Histological observation of masseter muscle fibers. A. Hematoxylin-eosin–stained masseter muscle fibers. Yellow border: masseter muscle fibers. B. Cross-sectional area of single masseter muscle fibers. The masseter muscle fibers in the BTX groups of all ages were atrophied compared with those in the CTRL groups of the corresponding age. Data indicate means ± SEM, n=5 independent experiments; *p<0.05, **p<0.01, ***p<0.001 compared to CTRL. Scale bar: 50 μm.
were significantly lower in each BTX group than in each CTRL group at all ages (Fig. 4).

**Histological observation of masseter muscle fibers**

The masseter muscle fibers in all the CTRL groups were packed closely together, with only narrow gaps between them (Fig. 5A). In contrast, the masseter muscle fibers in the BTX groups had wide gaps between them, and their cross-sectional areas were significantly lower in the BTX groups at each age compared with the corresponding CTRL groups (Fig. 5B).

**Histological observations at tendon insertion**

We observed Masson’s trichrome stained images of frontal sections from the third molar region of the heads of Wistar rats aged 10, 17, 24, and 31 weeks (Fig. 6A).

At all ages, the masseter was attached to the mandible by a tendinous insertion at the inferior margin of its ridge, and adhered to the mandible via the periosteum at all other points. In the 17-week-old, 24-week-old, and 31-week-old BTX groups, the boundary between the fibrocartilage and the mandible was unclear. In the BTX group, the number of fibrous chondrocytes at entheses was significantly lower than in the CTRL group at all ages (Fig. 6B).

Figure 6. Histological observations at tendon insertion. A. Masson’s trichrome stained YZ cross-sections of rat mandibles in the third molar region. M: masseter; Bo: bone (mandible); Te: tendon. Fibrous chondrocytes are observed (Yellow allows). The masseter is attached to the bone via the tendon and periosteum. B. Numbers of fibrous chondrocytes. In the BTX group, the number of fibrous chondrocytes at entheses was significantly lower than in the CTRL group at all ages. Data indicate means ± SEM, n=5 independent experiments; *p<0.05, **p<0.01, ***p<0.001 compared to CTRL. Scale bar: 50 μm.
Anisotropy of collagen fiber orientation

SHG images from the third molar region are shown in Fig. 7A. A multiphoton excitation phase-contrast microscope was used to detect imageable fibers as collagen fiber bundles. The orientation of collagen fiber bundles in the tendon with respect to the Z-axis was 99.3° (± 7.4°). Collagen fiber bundle diameter in 10-week-old and 17-week-old rats in the BTX group was significantly smaller than that in rats in the CTRL group (Fig. 7B). The collagen fibers in the bones of all groups were aligned in the direction of the tendon fibers. There was no significant difference between the diameter of collagen fiber bundles within bone in 24-week-old and 31-week-old rats in the BTX and CTRL groups.

**B**Ap crystal alignment

The preferential angle of alignment of **B**Ap crystals in entheses with respect to the Z-axis was 98.7° (± 8.5°). The X-ray diffraction intensity ratios at entheses calculated in the X-Y-Z three-axis system are shown in Figure 8. The X-ray diffraction intensity ratio of hydroxyapatite (HAp) powder in the reflecting system was 1.04, and that for HAp powder in the transmission system was 3.13. In the X-axis, the X-ray diffraction intensity ratio was significantly higher for 10-week-old and 17-week-old rats in the BTX group when compared with those in the CTRL group (Fig. 8A). In the Y-axis, the X-ray diffraction intensity ratio was significantly lower for 10-week-old and 17-week-old rats in the BTX group compared with those in the CTRL group (Fig. 8B).

However, there were no significant differences between 24-week-old...
and 31-week-old rats in the BTX and CTRL groups in either the X or Y-axis. In the Z-axis, the X-ray diffraction intensity ratio was lower than the value for HAp powder in the transmission system in both groups at all ages, exhibiting very low level of alignment (Fig. 8C).

**Discussion**

The bacterial metalloprotease contained in the BoNT/A formulation specifically inhibits the release of neurotransmitters at cholinergic nerve terminals, causing long-lasting muscle relaxation. Unlike invasive procedures such as masseter resection and masseter nerve transection, the use of this BoNT/A formulation minimizes the harm done to the muscle tissue. BoNT/A itself does not significantly affect the function of fibroblasts and chondrocytes and the formation of BAp crystals. Treating the masticatory muscle with BoNT/A formulation enables it to be maintained for long periods in an extremely high-quality state of reduced function. In this study, masseter muscle weight in the BTX group was lower than that in the CTRL group at all ages. Masseter muscle fibers also atrophied when BoNT/A was used. Tsai et al. and J.W. Choi et al. reported that reduced masseter muscle weight and masseter muscle atrophy induced a reduction in muscle functional pressure, and suggested that this reduction in load resulted in deformation of the mandible.

It may easily be envisaged that entheses are directly affected by reduced masseter functional pressure, the cause of such mandibular deformation. We investigated the micro/nanostructural characteristics of entheses of the masseter and its mechanical environment by means of a qualitative bone analysis. Chong et al. reported that fibrous chondrocytes are interposed in the tendon-bone insertion of the rat mandible. This finding suggested that if the persistent reduced muscle function due to BTX administration were to continue, insufficient fibrous chondrocytes would be induced, which might prevent the development of a healthy enthesis. In our bone quality analysis, we next investigated collagen fiber bundles within bone, which exhibit resistance to tensile stress. As reported by Yamada et al., belt-shaped collagen fiber bundles form within the bone as growth proceeds, and in the CTRL group these collagen fiber bundles in the bone at the masseter insertion were thick and aligned almost entirely consistently with the orientation of the tendon fibers. In rats treated with BTX during their growth and development, however, the diameter of collagen fiber bundles within the bone of growing rats was smaller than that in the CTRL group, whereas within the bone of mature rats there was no significant difference between them. Based on these findings, it was thought that persistent low masseter functional pressure during the growth and development phase leads to the generation of collagen fiber bundles within the bone at entheses that are deficient in both quantity and quality, but that in mature rats that have completed their growth and development, the collagen fiber bundles within the bone at entheses are less susceptible to the effect of reduced masseter functional pressure.

Our investigation of the preferential alignment of BAp crystals in the tendon insertion also found that it was extremely similar to the ori-
entation of collagen fiber bundles in the tendon in both groups at all ages. It has been reported that BAp crystal alignment exhibits resistance to compressive stress and the c-axis of BAp crystals is basically aligned with the orientation of collagen fiber bundles, a finding with which our results were generally in accord. BAp crystal alignment in the mandible basically exhibits single-axis dominancy in the mesial-distal direction (direction of the long axis), but directly beneath the tooth crowns the level of alignment with the mesial-distal direction decreases, and the crystals become predominantly aligned in the masticatory direction. It has been suggested that cortical bone tissue reflects the in vivo compressive stress distribution in the location of the bone concerned, and that this may change the preferential alignment direction. Bacon et al. reported that BAp crystals in the muscle insertion are preferentially aligned in a direction consistent with the orientation of the muscle.

In this study, we also found that in the mandibles of rats in the CTRL group the preferential alignment was consistent with the orientations of the muscle and tendon, but that in growing rats treated with BTX it was less closely aligned with the orientations of the muscle and tendon and became predominantly aligned in the mesial-distal direction.

Our results clarified that the BAp crystal alignment that would normally be achieved during growth and development was insufficiently obtained because of reduced masseter functional pressure due to BTX administration. In mature rats treated with BTX, however, the preferential alignment was consistent with the orientation of the muscle and tendon. It was thought that bone quality at entheses may be less susceptible to the effect of reduced masseter functional pressure in rats that have completed their growth and development. Our results in this study thus suggested that reduced muscle functional pressure during growth and development severely diminished qualitative factors of bone in entheses, reducing its mechanical function.

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Conflict of Interest

The authors have no COI exists.

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