Original

Effect of Zoledronic Acid on Bone Structure of the Mandible in Ovariectomized Mice

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Abstract: The objective of this study was to determine the effect of zoledronic acid on mandibular bone quality in osteoporotic model mice. Zoledronic acid was administered to ovariectomized mice, and mandibular bone was harvested. Polished specimens were prepared, and the biological apatite (BAp) crystal alignment and changes in collagen fiber bundles in the alveolar and basal regions of the mandible were analyzed. It was found that ovariectomy increased BAp crystal alignment. The administration of zoledronic acid post-ovariectomy normalized BAp crystal alignment in the basal region of the mandible. However, BAp crystal alignment in the alveolar region decreased significantly. Ovariectomy decreased the diameters of collagen fiber bundles in both the alveolar and basal regions and significantly increased their lengths. The administration of zoledronic acid post-ovariectomy decreased both the diameters and lengths of collagen fiber bundles. These results showed that the microarchitecture of the mandibular bone changes to compensate for osteoporosis-induced loss of bone mass and adapts to the load environment resulting from mastication. Interestingly, they suggested that zoledronic acid severely reduces the bone quality of osteoporotic alveolar bone in a site-specific manner.

Key words: Mandible, Bone structure, Zoledronic acid, Biological apatite crystal orientation, Collagen fiber

Introduction

Osteoporosis is defined by the World Health Organization (WHO) as “A disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk” [1]. The drugs currently used to treat osteoporosis and reduce the risk of fractures are bisphosphonates (BPs) and bone resorption inhibitors, including receptor activator of nuclear factor-κB ligand (RANKL) inhibitors and selective estrogen receptor modulators (SERMs) [2-4]. BPs are not only the most popular osteoporosis treatment drug, but they also have an excellent record in preventing bone metastasis of early breast cancer, and they are therefore widely used worldwide [5-8]. Paggiosi et al. reported that, although BPs increase the overall bone mineral density (BMD) of osteoporosis patients, there are significant differences in the degree of increase among bones [9]. BPs are known to have an effect on osteoporosis of the jawbone, increasing its overall BMD [10,11]. Takaishi et al. have also suggested that BPs may maintain or improve alveolar bone mass [12].

The National Institutes of Health (NIH) Consensus Development Conference held in 2000 declared that osteoporosis is “a skeletal disorder characterized by compromised bone strength predisposing to an increased risk of fracture” [12]. The declaration went on to make clear that the decrease in bone strength that leads to increased fracture risk is caused by reductions in both bone mass (BMD) and bone quality. This has led to the widespread recognition of the need to take into account bone quality factors including bone microarchitecture, bone turnover, microcracks, and the level of calcification of bone tissue when evaluating the state of osteoporosis. Conventional therapies to prevent the loss of bone mass are thus inadequate to prevent fractures, and priority is now placed on the importance of improving bone quality [13]. McLean et al. reported that osteoporosis-induced decreases in the bone quality of the femur increased the risk of fracture 3.8-fold in men and 1.9-fold in women [14]. Uusi-Rasi et al. found that the combination of BP administration and exercise therapy improved bone quality, as well as bone mass [15]. However, Bala et al. stated that the long-term use of osteoporosis drugs increases bone mass, but decreases bone quality, suggesting that bone strength may be lost [16]. Therefore, there is a difference of opinion on the effect of BPs on bone quality. Most studies of bone quality in osteoporosis have therefore focused on the bones of the trunk and limbs, and much remains unclear concerning the effect of BPs on bone quality in the jawbone.

In recent years, Nakano et al. have used materials engineering techniques to show that the alignment of biological apatite (BAp) crystals in bone is an important bone quality factor that affects bone strength [16]. Takano et al. have also shown that the anisotropy of collagen fiber bundles in bone varies greatly in different locations, and that this has a major impact on bone mechanical function [17]. Their reports demonstrated that BAp crystal alignment and collagen fiber bundle anisotropy are both important bone quality factors related to bone strength, the former in resisting compressive loading and the latter in resisting tensile loading. The objective of this study was to investigate the effect of BPs on bone quality in the jawbone by analyzing BAp crystal alignment and collagen fiber bundles in the mandible of mice administered a BP systemically.
Materials and Methods

Animals

The experimental animals were 18 eleven-week-old female C57BL/6J mice weighing approximately 25 g (n = 6/group). They were kept for 1 week preoperatively, then divided into three groups. Two of these groups underwent ovariectomy (OVX) at 12 weeks, and the third group underwent sham surgery (SHAM). Two weeks were allowed for postoperative wound healing, after which one of the ovariectomized groups was administered zoledronic acid (Zol)\textsuperscript{20}. All mice were euthanized at 5 weeks postoperatively, and their mandibles were harvested. The three groups comprised the OVX group of mice that underwent ovariectomy alone, the OVX+Zol group of ovariectomized mice administered Zol, and the SHAM group that underwent sham surgery. Drug administration was conducted by the injection of Zol (Novartis Pharma K. K., Tokyo, Japan: 1 mg/kg)\textsuperscript{21} into the dorsal skin under intraperitoneal triple anesthesia (midazolam/medetomidine hydrochloride/butorphanol tartrate) following the method of Mawardi et al.\textsuperscript{22} once weekly from 2 weeks postoperatively, for a total of two doses (Fig. 1). Mice in the SHAM group received a subcutaneous injection of physiological saline at the same site. These animal experiments were approved by the Tokyo Dental College Animal Experiment Committee (approval no. 206101).

Specimen preparation and designation of regions of interest (ROIs)

The mandibles were immediately fixed by complete immersion in 10% neutral phosphate-buffered formalin (PFA) for 2 days, after which they were transferred to 70% alcohol. The reference axes of the jawbones were designated as follows, following the method of Arakawa et al.\textsuperscript{23}: point a as the lowest point of the anterior thickened portion of the mandible; point p as the lowest point of the posterior thickened part of the mandible; the mandibular plane as the plane passing through lines a–a’ and p–p’; the Z axis as the axis passing through the midpoint of a–a’ and p–p’; the Y axis as the axis perpendicular to the mandibular plane; and the X axis as the axis perpendicular to the Y-Z plane (Fig. 2). The mandibles were embedded in unsaturated polyester resin (Rigolac, Nisshin E.M., Tokyo, Japan) taking the X, Y, and Z axes as references. The polymerized blocks were sliced along the XY plane containing the distal root of the mandibular first molar using a saw microtome with a 300-μm-wide blade (SP1600, Leica, Nussloch, Germany), and 100-μm-thick polished specimens were prepared. On these specimens, the alveolar bone ROI (A) was designated in the alveolar bone close to the lingual-side cervical part of the tooth, and the mandibular base ROI (M) was designated in the base of the mandible touching the mandibular plane (Fig. 3).

BAp crystal alignment measurements

X-ray diffraction intensity measurements were made in each ROI (Fig. 3). The analysis was conducted with a curved imaging-plate (IP) X-ray diffractometer (XRD: D/MAX RAPIDII-CMF, Rigaku Corporation, Tokyo, Japan). X-ray diffraction intensity measurements were made using both a transmission optical system and a reflecting optical system, with Cu-Kα rays as the radiation source in both cases. The tube voltage was 40 kV, and the tube current was 30 mA. The irradiation field was determined using the optical microscope fitted to the XRD (×0.6–4.8 magnification), and the incident beam was a circular microbeam with a diameter of 100 μm. The transmission optical system was used for measurements in the X- and Y-axis directions, and the reflecting optical system was used for measurements in the Z-axis direction, with the diffraction X-ray beam detected using the curved IP. The analysis conditions were those of the method of Nakano et al.\textsuperscript{18}. Using 2D Data Processing software (Rigaku Corporation), the X-ray intensity ra-

![Diagram of mandibular reference axes](image-url)
The ratios of the two diffraction peaks in the (002) and (310) planes were calculated from the diffraction ring.

**Analysis of collagen fiber bundles in bone using second-harmonic-generation imaging**

Second-harmonic-generation (SHG) images were acquired using a multiphoton confocal microscopy system (LSM 880 Airy NLO; Carl Zeiss Co., Ltd., 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 10x/0.8 M27; Carl Zeiss Co., Ltd.). The excitation wavelength for collagen fiber observation was 880 nm. ZEN imaging software (Carl Zeiss Co., Ltd.) was used for 3D construction of the image. Collagen fiber bundle tracing was carried out using high-resolution image analysis software (Imaris8.4, Bitplane AG, Zürich, Switzerland). Squares measuring 200 μm × 200 μm were designated from the ROIs in the alveolar and mandibular base regions, and the diameters and lengths of the visualized collagen fiber bundles were measured (Figs. 4 and 5).

**Statistical analysis**

EZR (The R Foundation for Statistical Computing version 2.13.0, Vienna, Austria) was used for statistical analysis, which was conducted using Tukey’s test, with \( p < 0.01 \) regarded as significant.
Results

**BAp crystal alignment**

In the alveolar region, a preferential uniaxial orientation in the Y-axis direction was observed in the SHAM and OVX groups. In this region, the X-ray diffraction intensity ratio in the Y-axis direction was significantly higher in the OVX group than in the SHAM group, whereas in the OVX+Zol group, the preferential orientation was lost, with no preferred alignment evident in any of the X, Y, or Z axes.

In the mandibular base, a preferential uniaxial orientation in the Z-axis direction was evident in all groups. The X-ray diffraction intensity ratio in the Z-axis direction of the mandibular base was higher in the OVX group than in the SHAM and OVX+Zol groups (Fig. 6).

**Analysis of collagen fiber bundles in bone**

SHG imaging showed that collagen fiber bundles were present in both the alveolar bone and the mandibular base in all three groups (Figs. 4 and 5). In both the alveolar region and the mandibular base, the collagen fiber bundles were finer and more fragile in the OVX group than in the SHAM group. In the OVX+Zol group, the collagen fiber bundles were finer and appeared more fragmented than in the OVX group.

A comparison of mean collagen fiber bundle diameter showed that it was significantly lower in the OVX and OVX+Zol groups than in the SHAM group in both the alveolar region and the mandibular base (Fig. 7). The diameter also tended to be lower in the OVX+Zol group compared with the OVX group, although this difference was not significant.
A comparison of mean collagen fiber bundle length showed that it was significantly higher in the OVX group and significantly lower in the OVX+Zol group compared with in the SHAM group in both the alveolar region and the mandibular base (Fig. 7). It was also significantly lower in the OVX+Zol group than in the OVX group.

**Discussion**

The results of the bone quality analysis in the present study showed that, in all three groups, BAp crystal alignment in the mandibular base exhibited a uniaxial preferential orientation in the Z-axis direction (the mesiodistal orientation of the alveolar bone). In the alveolar region, however, although a uniaxial preferential orientation in the Y-axis direction (the tooth axis direction) was evident in the SHAM group and OVX groups, no BAp preferential orientation was evident in the OVX+Zol group. Furuya et al. analyzed the alignment of BAp crystals in dentulous jawbones and reported that a preferential orientation in the mesiodistal direction was evident at the base of the mandible, whereas there was a uniaxial preferential orientation in the tooth axis direction in the alveolar region. The results for the SHAM group in the present study were similar to those of their report. Matsugaki et al. reported thatapatite crystals were preferentially oriented along the direction of collagen fibers in their invitro study. Ito et al. reported that collagen fibers and apatite crystals in rat femoral cortical bone were aligned along the long axis of the femur. In the present study, BAp crystal orientation showed uniaxial preferential orientation toward the Y-axis in the alveolar region and toward the Z-axis in the mandibular base. Therefore, it was suggested that the collagen fibrils, which serve as scaffolds for BAp crystals, may also show the same orientation. However, the X-ray diffraction intensity ratio in the Y-axis direction was significantly higher in the OVX group than in the SHAM group. Shiraishi et al. and Tanaka et al. reported that they observed an increase in bone quality in osteoporotic vertebral bone to compensate for loss of bone mass. Therefore, the greater BAp crystal alignment in the Y-axis direction seen in the OVX group may have been to maintain bone strength in compensation for lower bone mass. In addition, Shiraishi et al. and Tanaka et al. reported that BP administration to OVX animals improved the X-ray diffraction intensity ratio of vertebral body bones to the same level as that of the Sham group. In the present study, the crystal orientation of BAp in the mandibular base of the OVX+Zol group was not significantly different from that of the Sham group and was at the same level. Therefore, this suggests that the behavior of bone quality in the mandibular base may be similar to the changes in the trunk bone in response to osteoporosis and BP. However, the X-ray diffraction intensity ratio in the OVX+Zol group of alveolar regions was lower than that of the Sham group, and no preferential orientation was evident. The results suggest that, although the administration of BPs may have increased the bone mass of the alveolar bone, the c-axis orientation of BAp crystals was erratic, meaning that BP administration may have reduced bone quality.

The present analysis of collagen fibers showed that collagen fiber bundle diameter was significantly lower in the OVX group than in the SHAM group, but that collagen fiber bundle length was significantly higher. In the OVX+Zol group, the collagen fibers were fragmented, and only fine, short collagen fibers were present.

In a study by Bonnet et al. of OVX mice, BP administration greatly increased bone mass in both the jawbone and femur. However, animal experiments conducted by Kubek et al. and Allen et al. found that BP administration increased bone mass in the jawbone, although it severely inhibited bone remodeling. Wen et al. reported that jawbone has a higher affinity for BP than trunk bone, and Huja et al. reported that bone metabolic turnover in the alveolar region is six times higher than in trunk bone. Therefore, this suggests that the inhibitory effect of BP on bone remodeling may have been stronger in the alveolar region. The results of the present study also suggest that collagen fibers and BAp crystals failed to mature properly, although extensive bone formation in the alveolar region was associated with BP administration. Thus, these results demonstrated that the increase in jaw bone mass promoted by BP administration is genuine, but that bone quality, which is an important factor in bone strength, may be severely diminished in the jawbone in a site-specific manner.

The limitations of this study were the small number of samples and the fact that there was no analysis of other factors (microcracks, level of calcification, and bone turnover) involved in bone quality, although BAp crystal orientation and collagen fiber measurements are important factors in bone structure. A future research project will be to analyze bone strength using human samples for early diagnosis of osteoporosis from a dental perspective.

**Acknowledgements**

This study was supported by grants from the Private University Research Branding Project from MEXT of Japan (2019, 2020) and a research grant from the Japan Society for the Promotion of Science [Basic Research (C), grant number 18K09643].

The authors would like to express my appreciation to Dr. Masaaki Kasahara for his technical support.
Conflict of Interests
The authors have no conflict of interest to report.

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