Original

Effect of Bacterial Infection on Bone Quality and Structure in Osteonecrosis of the Jaw by Bisphosphonate (BP) Administration

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Abstract: Bisphosphonate (BP) formulations are drugs that improve bone strength by suppressing osteoclast activation, preventing fractures of the vertebrae and the femoral head, but their side effects include osteonecrosis of the jaw (ONJ). In this case, it is known as medication-related osteonecrosis of the jaw (MRONJ), and pathological and microbiological investigations have suggested that infection is one major causative factor. However, many points regarding the etiology of ONJ and its causative factors remain unclear. In this study, we administered BP to model mice and exposed their jaws to bacterial infection to produce a mouse model of BRONJ, and analyzed their bone structure, including an analysis of the quality of bone surrounding extraction cavities. We found that mice not exposed to bacterial infection did not develop ONJ, and that mice exposed to bacterial infection did not develop ONJ, but their bone mineral density was reduced compared with untreated mice. These results showed that although BP use increases bone mineral density, it reduces the alignment of collagen fibers and decreases bone quality. Zoledronate (Zol) alone resulted in epithelial healing, but reduced bone quality. In addition, it was suggested that bacterial infection could develop into a condition similar to BRONJ.

Key words: Medication-related osteonecrosis of the jaw (MRONJ), Osteonecrosis, Bacterial infection, Collagen fiber arrangement, Bioapatite crystal alignment

Introduction

Bisphosphonate (BP) formulations improve bone mineral density in the long bones and vertebrae, which contributes to the prevention of fragility fractures caused by osteoporosis and similar conditions. In the oral and maxillofacial region, however, BP can cause bisphosphonate-related osteonecrosis of the jaw (BRONJ), and the dental treatment strategy for patients taking BP formulations is the subject of vigorous debate. Cases of serious osteomyelitis of the jaw among patients prescribed bone resorption inhibitors for osteoporosis or malignancies such as breast cancer have also been reported in recent years. This condition is termed medication-related osteonecrosis of the jaw (MRONJ), and studies have confirmed that medications are a direct risk factor.

BRONJ is known to occur specifically in the mouth, and dental treatment involving invasion of the jaw and poor oral hygiene have been identified as risk factors. Hinson et al. pointed out that the environment of the jawbone makes it extremely susceptible to infection compared with bone in other parts of the body, and suggested that bacterial infection may play a major role in the development of BRONJ. However, not all patients taking bone resorption inhibition-related drugs develop BRONJ even if their oral hygiene is poor. Lo et al. reported that it develops in only 0.001-0.1% of patients taking bone resorption inhibition-related drugs. The oral environment is affected by numerous different factors, and previous analyses based on BRONJ patient data have found it extremely difficult to investigate individual factors as independent factors in detail. Park et al. produced BRONJ model mice in the effort to ascertain the etiology of ONJ, and showed that it is associated with a decline in the number of γδT cells. However, the association between bacterial infection and the development of BRONJ in these model mice has yet to be adequately studied.

In terms of the histological characteristics of osteonecrosis of the jaw in BRONJ, Bedogni et al. reported that the condition is characterized by the absence of any signs of bone remodeling in the osteonecrotic region. However, the pathophysiology of BRONJ is highly variable, and further investigation is required. As it was shown at the 2000 National Institutes of Health Consensus Conference that the strength of bone depends not only on bone mineral density but also on bone quality, researchers have been actively studying how qualitative factors, as well as quantitative factors, predict bone’s detailed mechanical function. Even bones with the same histological characteristics may differ greatly in their strength due to differences in microstructural and nanostructural characteristics such as bioapatite (BAp) crystal alignment and abnormal collagen fiber arrangement. The use of mechanical engineering techniques to investigate qualitative factors may make a major contribution to further understanding the pathophysiology of BRONJ.

In this study, we developed a mouse model of ONJ triggered by BP formulation and bacterial infection with the object of analyzing the pathology of BRONJ from the perspective of bone quality factors, and analyzed the histology and bone quality of the osteonecrosis site.
Materials and Methods

Animals

Fifteen C57BL/6j female mice (age 7 weeks, weight 24-25 g) were kept at a constant temperature in a 12-h light/dark cycle. Before and after tooth extraction they were fed regular rodent solid chow (MF solid chow, 12-mm-diameter pellets). This study was approved by the animal experiment committee of Tokyo Dental College (Ethics Application No. 197501).

Production of BRONJ model mice

The mice were allocated at random to three groups of five mice each: a group that underwent tooth extraction without BP administration (the Control group), a group that received zoledronate alone (Zol group), and a group that received both Zol and application of *Fusobacterium nucleatum* (Fn) (Fn+Zol group) (Fig. 1). Following Mawardi et al.'s method, the drug used was zoledronate 1 mg/kg (Novartis Pharma K.K., Tokyo, Japan), which was administered via subcutaneous injection in the back 2 weeks and 1 week before tooth extraction\(^2\). In the

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**Figure 1.** Zol group and Fn+Zol group procedures. In the Zol group, extraction was performed after Zol had been administered twice, and the mice were sacrificed on Day 15 and samples harvested. In the Fn+Zol group, Fn was applied to the extraction cavities using a micropipette on Days 2, 4, 6, 8, 12, and 14 after extraction.

**Figure 2.** Designation of region of interest (ROI). A. Designation of reference points, planes, and axes. Point a: Contact points between the upper and lower incisors. Point p: Occlusal contact points of the maxillary first posterior molars. Occlusal plane: Plane containing lines a-a' and p-p'. X-axis: Axis passing through midpoint of a-a' and p-p'. Y-axis: Axis perpendicular to occlusal plane. Z-axis: Axis perpendicular to X-Y plane. B. Designation of region of interest (ROI). The maxillary bone containing the extraction cavity of the maxillary first posterior molar was harvested en bloc (area enclosed by the black square), and measurements were made in an ROI measuring 100 μm x 100 μm with a point in the cortical bone 100 μm on the palatal side from the palatal root denoted as “palatal” (P) and a point 100 μm on the buccal side denoted as “buccal” (B).
Bone mineral density measurements

Bone density measurements were performed using a micro-CT system (HMX225 Actis4, Tesco Co., Tokyo, Japan) based on the three-axis system described above. The scanning conditions were as follows: tube voltage 140 kV, tube current 100 μA, matrix size 512 × 512, and voxel size 46.9 μm × 100 μm with a point in the cortical bone 100 μm on the palatal side of the extraction socket and the surrounding area every two days. Gram staining of thin slices prepared from the animals in the Fn group after their sacrifice demonstrated the invasion and persistence of Fn in the alveolar bone of all the specimens. The extraction cavities of the animals in each group were subsequently visually inspected immediately after extraction and on Days 5, 10, and 15, after which standardized photographs were taken and the epithelial defect rate around the extraction socket was calculated. The mice in all the groups were sacrificed on post-extraction Day 15. If an area was macroscopically observed to be covered with continuous mucosa, this was defined as “healed mucosa,” whereas areas not covered with continuous mucosa were defined as “mucosal defect,” and these were used to calculate the mucosal cover rate.

Specimen preparation and designation of region of interest (ROI)

The maxilla, including the extraction site, was harvested and fixed in 10% neutral buffered formalin solution (paraformaldehyde), after which it was transferred to 70% alcohol. A three-axis system was defined as follows. Point a: the points of contact between the maxillary and mandibular incisors; point p: the occlusal contact points of the maxillary first posterior molars; occlusal plane: the plane containing lines a–a’ and p–p’; X axis: the line passing through the midpoint of a–a’ and p–p’; Y axis: the line perpendicular to the occlusal plane; Z axis: the line perpendicular to the X–Y plane (Fig. 2A). The maxillary bone containing the extraction socket of the maxillary first posterior molar was harvested en bloc, and measurements were made in an ROI measuring 100 μm × 100 μm with a point in the cortical bone 100 μm on the palatal side from the palatal root denoted as “palatal” (P) and a point 100 μm on the buccal side denoted as “buccal” (B) (Fig. 2B).

Bone mineral density measurements

Samples were scanned with a micro-CT system (HMX225 Actis4, Tesco Co., Tokyo, Japan) based on the three-axis system described above. The scanning conditions were as follows: tube voltage 140 kV, tube current 100 μA, matrix size 512 × 512, and voxel size 46.9 μm × 46.9 μm × 50 μm. At the same time, 200 mg/cm² – 900 mg/cm² were also scanned and a calibration curve was calculated, after which the bone mineral density (BMD) of the ROI was measured with a TRI/3D-BON (RATOC System Engineering Co., Ltd., Tokyo, Japan).

Histopathological evaluation

Samples were decalcified in 10% ethylenediaminetetraacetic acid for one week and embedded in paraffin. Thin slices (4-μm thick) in the Y axis direction to the occlusal plane were prepared, hematoxylin–eosin (H-E) staining was carried out, and histopathological examination of the ROIs was conducted using an optical microscope (FSX100, Olympus Co., Ltd., Tokyo, Japan). All the bone lacunae were examined, and the number of osteocytes in each lacuna was measured to check whether or not osteonecrosis had occurred.

Qualitative analysis of collagen fiber bundles in bone

The thin slices on which histopathological examination had been conducted were then imaged by second-harmonic generation (SHG) imaging, and the collagen fiber bundles in the alveolar bone were examined and their diameters measured. A multiphoton confocal microscope system (LSM880 NLO, Carl Zeiss, Germany), an excitation laser (Chameleon Vision II, Coherent Inc., California, U.S.A.; wavelength 680–1080 nm, repetition rate 80 MHz, pulse amplitude 140 fs) and an objective lens (Plan-Apochromat 20x / 0.8 M27; Carl Zeiss, Germany) were used for SHG imaging. The excitation wavelength was set at 880 nm for collagen fiber observation. Image acquisition, orthogonal view processing, and trimming were carried out with ZEN Black (Carl Zeiss). Collagen fiber bundle diameter was analyzed using image analysis software (IMARIS; Carl Zeiss, Germany). Collagen fiber bundles were defined as collagen fibers of diameter ≥ 1 μm, and the mean diameter of collagen fiber bundles in P and B of the ROI was calculated.

Quantitative evaluation of BAp crystal alignment

X-ray diffraction intensity was measured at points P and B (Fig. 2B). An analysis was performed using an optical-system curved-imaging-plate X-ray diffraction (XRD) system (D/MAX RAPIDII-CMF, Rigaku Co., Japan). X-ray diffraction intensity measurements were made using both a transmission optical system and a reflection optical system, using Cu-Kα radiation as the radiation source in both cases. The measurement conditions were as follows: tube voltage 40 kV and tube current 30 mA. The irradiation field was set using the optical microscope belonging to the XRD system (×4.6-4.8), and the incident beam was set as a microcircle 100 μm in diameter. Measurements were made in the X-axis direction using the reflection optical system and in the Y and Z axis directions using the transmission optical system, and the diffraction X-ray beam was detected on a curved imaging plate (IP). The analysis conditions were those used by Nakano et al. X-ray diffraction intensity ratio of the diffraction peaks in the (002) and (310) planes was calculated from the diffraction ring images using 2D data processing software (Rigaku Co., Japan).

Statistical analysis

A Mann-Whitney U test was used for comparisons between two sets of data from different samples, and a Bonferroni test was used for multiple comparisons. All statistical analysis was conducted using EZR version 2.13.0 (the R Foundation for Statistical Computing, Vienna, Austria).

Results

Macroscopic findings of epithelial healing after extraction in each group

Fig. 3 shows the macroscopic findings of the extraction cavities and surrounding tissue on Day 15 after extraction. In the Control and Zol groups, the extraction cavities were completely covered with epithelium. In the Fn+Zol group, they were still not covered by epithelium on Day 15, and the bone remained exposed. The epithelial defect rates at all the ROIs calculated from the standardized photographs also showed that the epithelium was completely closed in the Control and Zol groups on Day 10, whereas in the Fn+Zol group epithelial defects persisted even after 15 days had elapsed.
Figure 3. Intraoral photographs of the mouse palate and mucosal cover rate around the extraction cavity. The top row shows intraoral photographs from the different groups, and the lower row the mucosal cover rates. In the Fn+Zol group, the cavity was not covered by mucosa at all and the bone was constantly exposed.

|       | Day 0(%) | Day 5(%) | Day 10(%) | Day 15(%) |
|-------|----------|----------|-----------|-----------|
| Control(n=5) | 0        | 20       | 100       | 100       |
| Zol(n=5)    | 0        | 40       | 100       | 100       |
| Fn+Zol(n=5) | 0        | 0        | 0         | 0         |

Figure 4. Histopathological images of the extraction cavity and surrounding tissue. The top row shows low-magnification images, and the bottom row shows high-magnification images of points P and B. In the Fn+Zol group, there were numerous empty bone lacunae at both P and B. EP: epithelium. SOC: extraction socket (the region inside the black line). Black squares: measurement points. Black arrows: empty bone lacunae.
Bone Mineral Density

The mean BMD of the Control group was 795.9 ± 31.8 mg/cm³. In the Zol group and the Fn+Zol group it was 845.1 ± 22.7 mg/cm³ and 833.6 ± 21.2 mg/cm³, respectively, both significantly higher than the value in the Control group (Table 1).

| Group     | n  | BMD (mg/cm³)       |
|-----------|----|--------------------|
| Control   | 5  | 795.9±31.8         |
| Zol       | 5  | 845.1±22.7         |
| Fn + Zol  | 5  | 833.6±21.2         |

* p<0.05

Figure 5. Proportion of empty bone lacunae in the ROI in each group. Compared with the Control and Zol groups, the proportion of empty bone lacunae was significantly higher in the Fn+Zol group.

Figure 6. H-E staining and SHG imaging. A. H-E staining images of the extraction sockets and surrounding tissue (top row) and SHG imaging of collagen fiber bundles (bottom row). At points P and B in the Control and Zol groups, collagen fiber bundles ran parallel to each other toward the extraction socket. However, in the Fn+Zol group the courses of collagen fiber bundles were not apparent. SOC: extraction socket (the area inside the black or white broken line). Black and white squares: ROI. Green: collagen fiber bundles. Red: other tissue. B. High-magnification histopathological images of the ROI (top row) and SHG imaging of collagen fiber bundles at the same site (bottom row).
Like the macroscopic observations, H-E staining also revealed that the extraction cavities in the Control and Zol groups were covered by epithelium, whereas in the Fn+Zol group the epithelium had not regenerated and the bone was exposed (Fig. 4). Callus formation in the extraction sockets was evident in the Control and Zol groups, but was almost absent from the Fn+Zol group. Inflammatory cell infiltration was also particularly prominent around the necrotic alveolar bone in the Fn+Zol group (Fig. 4). The proportion of empty bone lacunae in the ROI was significantly higher in the Fn+Zol group than in both the Control and Zol groups (Fig. 5).

**Histopathological evaluation**

**Figure 7.** Diameters of collagen fiber bundles in the Control, Zol, and Fn+Zol groups. In both the Zol and Fn+Zol groups, the diameters of the collagen fiber bundles were significantly lower than those of the Control group.

**Figure 8.** X-ray diffraction intensity ratios in the three axes. The ratios were significantly lower in the Fn+Zol group than in the Control and Zol groups in all three axes.

**Qualitative analysis of collagen fiber bundles in bone**

Observations of the collagen fiber bundles in the ROI visualized on SHG imaging revealed large numbers of collagen fiber bundles in and around the extraction cavities in both the Control and Zol groups (Fig. 6A). However, no collagen fiber bundles were seen in the bone of the Fn+Zol group. In the Control and Zol groups, collagen fiber bundles ran parallel to one another toward the extraction socket (Fig. 6B). A comparison of the mean collagen fiber bundle diameters showed that they were significantly lower in both the Zol and Fn+Zol groups than in the Control group (Fig. 7).
BAP crystal alignment

The X-ray diffraction intensity ratio at each measurement point was significantly lower in the Fn+Zol group in the X-axis, Y-axis, and Z-axis directions than it was in the Control and Zol groups (Fig. 8).

Discussion

Akita et al. reported that the continuous administration of Zol to rats increased the BMD of the maxilla as well as of bones in the trunk and limbs20. In this study, we similarly found that alveolar bone in mice treated with Zol had a high BMD, not only in mice given Zol alone but also those to which Fn was applied.

In mice treated with Zol alone, the extraction cavities were already completely covered by epithelium within one week and there was no exposed bone, whereas those both treated with Zol and infected by Fn exhibited poor epithelial healing and ONJ. Mawardi et al. suggested that continuous administration of a BP formulation during Fn infection may suppress the production of keratinocyte growth factor (KGF), an epithelial growth factor21. The condition developed by mice in the Fn+Zol group in this study resembled their report that not only was the healing of mouse maxillary epithelium delayed but that epithelial regeneration was also suppressed.

Our analysis of bioapatite crystal alignment and abnormal collagen fiber arrangement in bone showed that there were significant differences in bone quality between the Control group and both the Zol and Fn+Zol groups. Nakano et al. suggested that BAP crystals are basically oriented alongside collagen fiber bundles in bone, and that they may optimize in bone quality between the Control group and both the Zol and Fn+Zol groups. Nakano et al. reported that high-dose Zol administration blocks collagen stability in the bone recovery process, delaying healing20. In this study, we also found that mice given a high dose of Zol had smaller collagen fiber bundle diameters than those in the Control group. In the Fn+Zol group, the collagen fiber bundle diameters were even more significantly lower than those in the Zol group. Our results in this study suggested that high-dose Zol administration when infection is present may greatly hinder collagen fiber production in bone, sometimes resulting in the failure of collagen fiber bundle generation itself. If insufficient collagen fiber bundles have been generated, the alignment of bioapatite crystals, which are deposited along collagen fibers, will be similarly poor. In this study, it was suggested that the pathogenesis of BRONJ was not only BP but also bacterial infection was an accelerator factor, and the pathology was accompanied by low bone quality. Marx defines a bisphosphonate-related condition as avascular osteonecrosis11. Furthermore, it has been reported that the angiogenesis inhibitory effect of BP administration is greatly related to the development of BRONJ. However, angiogenesis differs in humans and mice, and as mice do not possess Haversian canals, no clear blood vessels were visible, which is not the case in humans. We therefore felt that there are limits to evaluation using model mice. Osteon structures form in humans, which is not the case in mice. Humans possess a Haversian remodeling mechanism in which bone resorption by osteoclasts results in the formation of bone matrix with a concentric lamellar structure that encloses the blood vessels along the long axis of the bone. To date there have been almost no studies of the relationship between vascular structures and bone matrix formation in the cortical bone of mice, rats, and other small animals that lack Haversian remodeling, although a strong structural association between vascular networks and bone matrix orientation has been identified in mouse femur. We intend to conduct further studies of the courses of blood vessels.

ONJ is only a concept up to Stage 0, and the addition of an accelerant factor called bacterial infection is an important key to up staging. Our results suggested that the presence of bacterial infection as an accelerating factor may be an important key to the up-staging of BRONJ, and that this condition may progress to Stage 2 or 3 as a result of bacterial infection and host response.

Conflict of Interest

The authors have declared COI exists.

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