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Research Paper
Bone Biology

Molecular alterations of newly formed mandibular bone caused by zoledronate

Y. Yoshioka[1], E. Yamachika[2], M. Nakanishi[3], T. Ninomiya[4], K. Nakatsuji[1], M. Matsubara[1], N. Moritani[1], Y. Kobayashi[4], T. Fujii[3], S. Iida[1]

[1]Department of Oral and Maxillofacial Reconstructive Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama City, Japan; [2]Department of Oral and Maxillofacial Reconstructive Surgery, Okayama University Hospital, Okayama City, Japan; [3]Department of Applied Chemistry, Graduate School of Natural Science and Technology, Okayama University, Okayama City, Japan; [4]Division of Hard Tissue Research, Institute for Oral Science, Matsumoto Dental University, Shiojiri, Nagano, Japan

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Abstract. Bone quality is defined by structural and material characteristics. Most studies on the mandible have focused on the analysis of structural characteristics, with insufficient investigation of material characteristics. This study tested whether zoledronate affects the material characteristics of newly formed mandibular bone. Thirty-six female Wistar rats were assigned to three groups: sham-ovariectomized rats (SHAM, $n = 12$), ovariectomized rats (OVX, $n = 12$), and ovariectomized rats treated with zoledronate (ZOL, $n = 12$). The left side of the mandibular ramus of all rats was drilled bicortically. Twenty-eight days after surgery, all surviving rats were euthanized and all mandibles were removed. Raman microspectroscopy was performed, and five spectra per specimen of newly formed mandibular bone were analysed. Compared with OVX rats, the mineral/matrix ratio in ZOL rats was significantly increased (5.43 ± 1.88 vs. 7.86 ± 2.05), while crystallinity (0.055 ± 0.002 vs. 0.050 ± 0.002), relative proteoglycan content (0.43 ± 0.10 vs. 0.31 ± 0.05), and collagen structural integrity (1.16 ± 0.21 vs. 0.72 ± 0.06) were significantly decreased. These changes in material characteristics may explain why rats that received zoledronate exhibited peculiar biological phenomena such as bisphosphonate-related osteonecrosis of the jaw.

Key words: Raman spectroscopy; bisphosphonate; bone quality; mandible; osteonecrosis.

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Bone strength is determined by bone density and bone quality. Bone quality is defined by its structural and material characteristics. Structural characteristics include bone organization and size, as well as the trabeculae of cancellous bone and porosity of cortical bone. Material characteristics include the degree of mineralization, mineral quality, and matrix quality, which is determined by the proteoglycan content and the structural integrity of bone collagen[1,2].

Bisphosphonates are currently the first-line treatment for osteoporosis. The influence of bisphosphonates on bone density and bone quality in the context of maxillofacial surgery has attracted attention[3–5]. For example, it is suspected that the underlying cause of medication-related osteonecrosis of the jaw after surgical procedures is the deterioration of bone quality induced by bone resorption inhibitors like bisphosphonates[6]. Accordingly, the importance of treatment that improves the quality of mandibular bone has been recognized. The mandible has a characteristically higher remodelling rate than

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other skeletal bones. In addition, the concentration of bisphosphonate in the mandible can be 100 times higher than that in other skeletal bones[7]. This potentiates the effect of bisphosphonates, resulting in a stronger inhibition of bone remodelling compared with other skeletal bones[8].

Bone abnormalities occur not only because of an abnormality in bone remodelling but also because of abnormalities of material characteristics such as increased oxidant or glycation stress associated with aging or decreased sex hormones[10–12]. Raman microspectroscopy has become a powerful tool for the assessment of the material characteristics of bone[13]. This technique analyzes information based on molecular vibration, which allows non-contact and non-destructive analysis of the molecular structure and the assessment of crystallinity. Furthermore, studies using Raman microspectroscopy have reported a high degree of mineralization and changes in collagen quality in skeletal bones induced by bisphosphonates[5,10]. However, the influence of bisphosphonates on the material characteristics of newly formed mandibular bone that is in the process of healing remains unknown.

It was hypothesized that bisphosphonates potentially affect the material characteristics of newly formed mandibular bone that is in the process of healing after an invasive procedure. In this study, Raman microspectroscopy was used to evaluate changes in various material characteristics of newly formed bone, including mineralization, crystallinity, carbonate content, proteoglycan content, and collagen structural integrity, in the mandibles of rats that had received zoledronate.

Materials and methods

Animals

A total of 36 female Wistar rats (ovariectomized rats, $n = 24$; sham-ovariectomized rats, $n = 12$) aged 8 weeks were obtained from Japan SLC, Inc. (Hamamatsu, Shizuoka, Japan). Ovariectomized or sham-ovariectomized rats were operated on 4 days before arrival in the study facility. The rats were housed in a temperature and humidity-controlled room with a 12-hour day and night cycle and had access to food and water ad libitum. After acclimatization for 18 days, the rats were assigned to three groups: (1) ovariectomized rats treated with zoledronate (ZOL rats, $n = 12$); (2) sham-ovariectomized rats treated with vehicle (normal saline) (SHAM rats, $n = 12$); (3) ovariectomized rats treated with vehicle (normal saline) (OVX rats, $n = 12$).

Dosing regimens and surgical technique

After acclimatization for 18 days, the rats received the assigned study medication only once. ZOL rats were administered a single subcutaneous dose of zoledronate at 120 μg/kg body weight (equivalent to the dose used for the treatment of human osteoporosis)[14], and OVX and SHAM rats were administered an equivalent volume of saline subcutaneously. One rat in the ZOL group died suddenly 1 day after zoledronate administration and was therefore excluded from the study. Two days after administration, all rats underwent the study surgical procedures under general anaesthesia (medetomidine, midazolam, and butorphanol mixture)[15]. The left side of the lateral aspect of the mandibular ramus was incised to the sub-periosteal level, and sub-periosteal peeling to the lower and posterior margins of the mandible was also performed to clearly reveal the surgical field. A point located 2 mm from the lower and posterior margins on the left side of the mandible was drilled bicortically with a 1-mm round bur. The wound was closed with sutures.

Twenty-eight days after surgery, all rats were euthanized under general anaesthesia (medetomidine, midazolam, and butorphanol mixture)[15]. The right femur and mandible were removed and stored in 70% ethanol at 4 °C for micro-computed tomography (micro-CT), peripheral quantitative computed tomography (pQCT), and Raman analysis.

Analysis of the right femur using micro-CT

The undecalcified right femurs (12 from SHAM rats, 12 from OVX rats, and 11 from ZOL rats) were subjected to three-dimensional (3D) micro-CT analysis (Scan Xmate-A080; Comscan Tecno Co. Ltd, Yokohama, Japan). Once these CT images had been constructed into 3D images, bone morphometric analysis was performed using analytical software (TRI/3D-BON; Ratoc System Engineering Co. Ltd, Tokyo, Japan). The trabecular bone in the distal metaphysis of the femur was chosen as the sampling site. The site was located 1.5–3.5 mm from the growth plate, and morphometric parameters included bone volume (BV/TV, %), trabecular thickness (Tb.Th, μm), trabecular number (Tb.N, 1/mm), and trabecular separation (Tb.Sp, μm).

Analysis of the right femur using pQCT

The undecalcified right femurs (12 from SHAM rats, 12 from OVX rats, and 11 from ZOL rats) were scanned by pQCT, and bone mineral density (BMD, mg/cm^3) was measured. The points at 1 mm, 2 mm, and 3 mm to the diaphysis from the growth plate were defined as regions 1, 2, and 3, respectively. The BMD in these regions was measured by pQCT (Norland/Stratec XCT Research SA+; Stratec Medizintechnik GmbH, Pforzheim, Germany).

Analysis of the mandible using pQCT

A large area with a diameter of 0.8 mm surrounding the centre of the drilling area on the mandible (12 SHAM rats, 12 OVX rats, and 11 ZOL rats) was scanned by pQCT, and the BMD (mg/cm^3) was measured.

Analysis of the mandible by Raman microspectroscopy

The undecalcified mandibles (12 from SHAM rats, 12 from OVX rats, and 11 from ZOL rats) were subjected to Raman microspectroscopy (NRS-5100; Jasco Corporation, Tokyo, Japan). The Ar-ion laser power (wavelength 532 nm) for all measurements was 100 mW. The Raman spectra were obtained with an acquisition time of 60 s twice and accumulated. The analysis was confined to the Raman scattering region between 800 cm^{-1} and 1800 cm^{-1}. Quantitative maps of the newly formed bone phase distribution within the sample area (300 μm × 120 μm) were recorded, employing a scanning step size of 10 μm. In addition, the outer circumference at a distance of 100 μm from the outer periphery of the drilling area was examined with an optical electron microscope attached to the Raman microspectroscope. Five spectra per specimen were then obtained randomly from the entire circumference. Peak assignment and interpretation were conducted according to the methods described in the literature[16–18]. For each measurement spot, commonly used Raman bone metrics were calculated: (1) the mineral/matrix ratio, which is the intensity ratio between υ_1PO_4 (930–980 cm^{-1}) and amide I (1620–1700 cm^{-1}); (2) crystallinity, which is the inverse of the full-width at half-height (FWHH) of the υ_1PO_4 band; (3) B-type carbonate substitution, which is the intensity ratio between B-type CO_3^{2-} (1050–1115 cm^{-1}) and υ_1PO_4 bands; (4) relative proteoglycan content (proteoglycan/organic matrix ratio), which is the intensity

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