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ORIGINAL ARTICLE

Imaging and histomorphometric evaluation of mandible and tibia of rats treated with bisphosphonates

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Abstract

Objectives To evaluate the mandible and tibia of rats treated with bisphosphonates (BPs) by imaging and histomorphometric analysis.

Study design Thirty-four rat specimens (*Rattus norvegicus*, Wistar strain) were distributed into 3 groups: (1) 12 rats treated with zoledronic acid; (2) 12 rats treated with clodronate; and (3) the control group, containing 10 rats that received saline. All bones were exposed to cone beam computed tomography (CBCT). The images were analyzed to determine bone density (BD), using the software OsiriX 7.0. Histological slides were prepared from the specimens and the proportion of bone volume (BV) was quantified using the software Adobe Photoshop CC.

Results There was no statistically significant difference in BD either between the drug groups or between mandible and tibia. BV between BPs and control group did not show a significant difference. However, comparing the two bones, the mandibles in the control group displayed higher BV than did the tibiae in the same group.

Conclusion According to our results, we conclude that (1) BD was not altered by bone type or by type of BP administered, and (2) treatment with zoledronic acid or clodronate did not affect BV in the mandible or tibia of test groups.

Keywords Bisphosphonate · Cone-beam computed tomography · Jaws · Bone tissue · Bisphosphonate-associated osteonecrosis of the jaw

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Introduction

Bisphosphonates (BPs) are drugs that inhibit bone resorption through direct and indirect effects on osteoclasts, which undergo apoptosis or become unable to differentiate from hematopoietic stem cells [1–4]. Because of this antiresorptive effect, BPs are used to prevent and treat bone diseases, characterized by increased osteoclast activity, such as osteoporosis, osteogenesis imperfecta, Paget's disease, multiple myeloma, and bone metastasis from malignant tumors [3–6]. The nitrogen-containing BPs (N-BPs) have a higher pharmacological potency and exert their action by inhibiting the mevalonate biosynthetic pathway, affecting the production of signaling molecules that modulate osteoclast (OC) function. The non-nitrogen-containing BPs (non-N-BPs), on the other hand, are metabolized into cytotoxic ATP analogs that accumulate inside OCs. Thus, BP therapy alters bone metabolism and reduces bone resorption, due to the interference and/or inhibitory effects on cells involved in this physiological proccess, especially the OCs [2, 5–7].

An injurious outcome associated with BP treatment is known as medication-related osteonecrosis. Marx, in 2003, was the first to describe BP-related osteonecrosis of the jaws (BRONJ) as a side effect of BP therapy [8]. In 2014, the American Association of Oral and Maxillofacial Surgeons (AAOMS) updated the nomenclature, due to reports of other antiresorptive agents (such as denosumab) and antiangiogenic drugs (such as sunitinib and bevacizumab) associated with this disorder, which was renamed as medication-related osteonecrosis of the jaws (MRONJ) [6, 9, 10]. The condition's etiology seems to be multifactorial, and clinically, it most commonly develops after dentoalveolar surgery or another kind of trauma, although a spontaneous occurrence is possible [5, 10].

The diagnosis of MRONJ is based on the clinical examination and imaging tests, combined with the patient's medical history [10, 11]. Imaging techniques play an essential role not only in inferring disease progression but also in detecting MRONJ early and tailoring therapeutic management for each patient [12, 13]. Advanced imaging procedures such as computed tomography (CT), cone beam CT (CBCT), and positron emission tomography (PET)-CT may be more practical for detecting lesions, compared to conventional imaging such as panoramic radiography [13]. Microcomputed tomography (microCT) has been widely used for high-quality structural analysis of bone, teeth, and other materials. However, high cost and radiation dose limit the use of this modality [14].

A number of in vivo studies have demonstrated the effects of BPs in bone tissue following procedures [4, 15–18]. Nevertheless, there is a lack of data about the comparison between different types of bones and between antiresorptive drugs. Hence, the objective of the present study was to compare, by means of imaging and histomorphometric parameters, the mandible vs. tibia, and N-BP (zoledronic acid) vs. non-N-BP (clodronate), to investigate possible differential effects on bone structure.

Methods

Ethical approval

The study was approved by the Ethics Committee for Animal Use of the Pontifical Catholic University of Rio Grande do Sul (CEUA/PUC-RS) in April 2009 (Approval No. 09/00083), and the procedures were carried out in accordance with the institutional guidelines for animal care and use.

Sample

A total of 34 adult female rats (*Rattus norvegicus*, Wistar strain) were included, which had a mean age of 120 days and a mean weight of 230 g [4]. The model size was based on prior studies by Maahs et al. (2011) and Vasconcelos et al. (2012), which used comparable methods [4, 11]. The animals were randomly allocated to 3 groups, accordingly with the BP used: (1) zoledronic acid (ZA) group—12 animals were treated with the N-BP ZA (Novartis Pharma AG, Basel, Switzerland) intraperitoneally (i.p.) (0.6 mg/kg, every 28 days) [4, 16, 19]; (2) clodronate (CL) group—12 animals treated with the non-N-BP CL (Jenahexal Pharma GmbH, Thuringia, Germany), i.p. (20 mg/kg, every 28 days) [4]; and (3) control group—10 animals received saline (0.9% sodium chloride). As the intravenous injection and the capacity to manage large amounts of fluids is complication in laboratory rodents, intraperitoneal administration is frequently used. In addition, it decreases the risk of adverse effects and provides less discomfort to the animal [20].

The rats were euthanized by deep anesthesia with isoflurane (Cristalia, Porto Alegre, RS, Brazil) in an appropriate anesthesia compartment, after 120 days of drug administration. The specimens were examined using a No. 5 clinical probe (SS White, Duflex, Rio de Janeiro, RJ, Brazil) to determine the presence/absence of macroscopic bone lesion. Afterwards, the mandibles and left tibiae were dissected and fixed for 24 h in 10% buffered formalin (TopGlass, Porto Alegre, RS, Brazil).

CBCTs

The samples were submitted to CBCT, using a Prexion 3D® tomograph (Terarecon, San Mateo, CA, USA). Mandibles and tibiae were placed in plastic boxes, each composed of three 2.5 × 6.0 cm compartments containing 1 mandible or 1 tibia each, immersed in formalin to attenuate radiation. The boxes were positioned in the machine with the aid of polystyrene boxes, to acquire an ideal height for the tomographic scanning. The following technical parameters were applied: 90 kVp, 4 mA, voxel resolution 0.1 mm, 37 s, and a 56 × 52-mm field of view (FOV). The images obtained were stored on a hard drive in Digital Imaging and Communications in Medicine (DICOM) format.

Tomographic analysis

The tomographic images were visualized and evaluated by a calibrated and blinded examiner using the Osirix 7.0 software (OsiriX, http://www.osirix-viewer.com). Multiplanar Reconstructions Screen (MPR Screen) visualization was used to investigate the sagittal, coronal, and axial planes and to define the region of interest (ROI). For determination of mandibular ROI, the images were manipulated so the sagittal axis was placed in the center of the long axis of the left side of the mandible, parallel to the cortical bone. The axial axis was positioned next to the incisor root. For each mandible, an anterior and a posterior ROI were determined, generating a double sample number for each group. For the anterior ROI, the coronal axis was placed where the bone tissue started to circle the left incisor root, and the bone density (BD) measures were made in the bone superior to the root (Fig. 1a). For the posterior ROI, the coronal axis was moved to the distal region of the last molar on the left side, measuring the BD posteriorly to the molar root (Fig. 1b). For the establishment of ROI in tibia, the images were manipulated so the sagittal and coronal axes were positioned over the center of the long axis, and the axial axis placed next to the midpoint of the long axis. The BD was measured in the superior and inferior regions of cortical bone, in the central part of the diaphysis (Fig. 1c). The BD values were obtained from the sagittal plane image for all ROIs, using the Point tool, in Hounsfield units (HU). For each region, two consecutive slices were analyzed, measuring 3 points in each anterior and posterior slice of mandible, and 6 points for each tibia slice (3 for the superior cortical and 3 for the inferior cortical).

Finally, the mean BD value for the mandible was obtained by combining the anterior and posterior region of each specimen, and by combining the superior and inferior cortical for each tibia.

Histological processing

All mandibles and tibiae were decalcified in 10% nitric acid with urea after tomographic exposure. The mandibles were hemi-sectioned, and the left side was selected for analysis, as in the tomography examination. Subsequently, an anteroposterior section was made anterior to the first molar, resulting in an anterior and a posterior fragment, which was paraffin-embedded separately. The tibiae were sectioned at the midpoint of the sagittal axis, and each fragment was paraffin-embedded individually. The paraffin blocks were cut into 4-μm sections and stained with hematoxylin and eosin (H&E).

Histomorphometry

Sixty-eight histological slides were obtained from the 34 mandibles. For the tibiae, 34 slides were obtained. The sections were digitized using a Leica DM3000 light microscope (Leica Microsystems GmbH, Wetzlar, Germany), connected to a Leica DFC7000 T camera (Leica Microsystems GmbH, Wetzlar, Germany) and an AMD Phenon II X4 3.4 GHz computer with Leica Application Suite software (Leica Microsystems GmbH, Wetzlar, Germany). The images were captured using a × 10 objective and stored in TIFF (Tagged Image File Format). For each slide, as many fields as necessary were captured to cover all the bone tissue in the ROI. In total, 255 images were obtained for the mandibles, and 710 images for the tibiae.

A calibrated and blinded examiner, using the software Adobe Photoshop CC (Adobe Systems, San Jose, CA, USA) analyzed the images. The bone tissue present in each field was selected, using selection tools, and quantified using the pixel value of the Histogram [21]. Blood vessels, medullary spaces, empty spaces, and cartilage were removed from the selection.

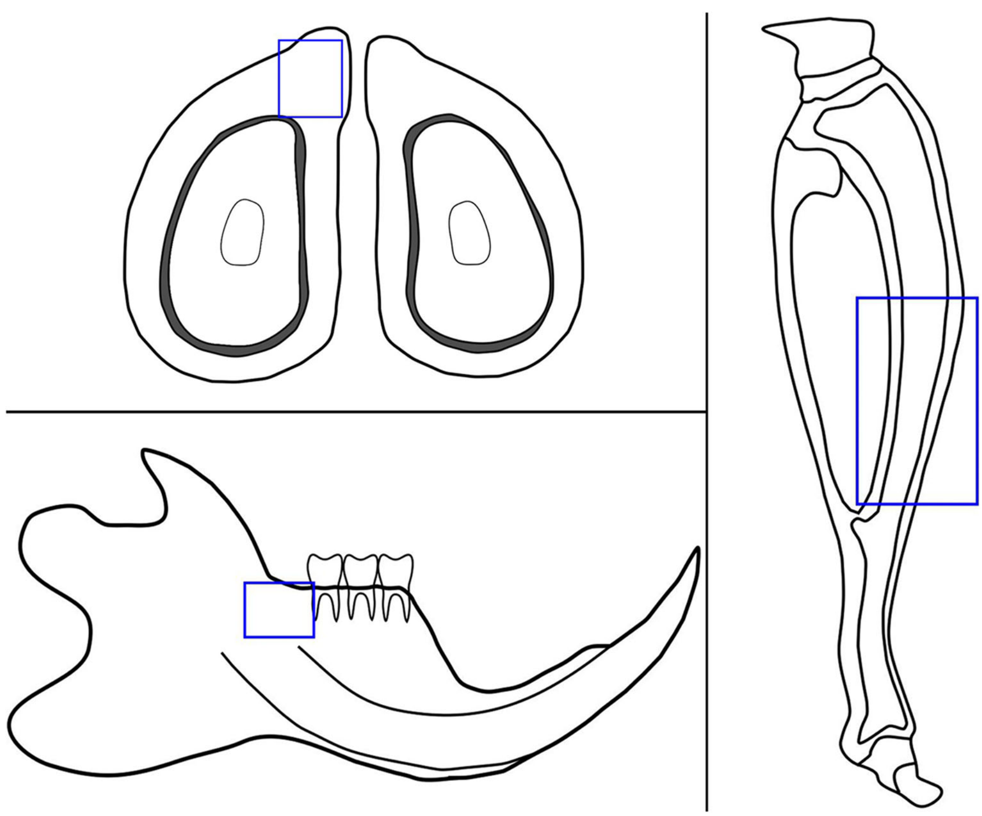

Fig. 1 Schematic diagram of BD quantification. **a** Mandibular anterior ROI. **b** Mandibular posterior ROI. **c** Tibial ROI

Next, considering the mean pixel value for each specimen, a bone volume (BV) proportion was obtained, corresponding to the proportion of total image area occupied by bone tissue (Fig. 2) [2, 21].

Data analysis

Ten random tomographic images were used for the examiner calibration for tomographic analysis. For histological analysis, 20 random histological images, twice at different times, were utilized. The results of these two distinct calibrations were subjected to a paired *t* test and Pearson's correlation coefficient, showing no significant difference ($p > 0.05$) and a strong correlation ($r > 0.9$). The comparison between the BPs and control group, for each bone, was done by means of ANOVA, at a significance level of 5%. Student's *t* test was used to compare the different bones, mandible, and tibia, with a significance level of 5%. The statistical analysis was carried out with the Stata 15.0 software (StataCorp, College Station, TX, USA).

Results

Macroscopic evaluation

On macroscopic examination, neither mandibles nor tibiae exhibited visible bone lesions.

Tomographic analysis

The results of the tomographic evaluation of BD are shown in Table 1.

Mandible

No significant differences were found in the BD values between ZA, CL, and control groups ($p = 0.419$), with the anterior and posterior regions combined.

Tibia

No significant differences were observed between the ZA, CL, and control groups ($p = 0.871$).

Mandible vs. tibia

Comparing the two bones in each group, no significant differences were observed for the ZA ($p = 0.449$), CL ($p = 0.602$), or control ($p = 0.487$) group.

Histomorphometry

The results of histomorphometric evaluation of BV proportion are shown in Table 2.

Mandible

There were no significant differences in BV proportion between ZA, CL, and control groups ($p = 0.939$), with the anterior and posterior regions combined.

Tibia

No significant differences were found between ZA, CL, and control groups ($p = 0.698$).

Mandible vs. tibia

Although the mandible exhibited a higher proportion of BV in all groups, the comparison of the two bones showed no significant differences for ZA ($p = 0.056$) and CL ($p = 0.089$) groups. For the control group, a statistical difference was found ($p = 0.032$), with a higher proportion in the jaw.

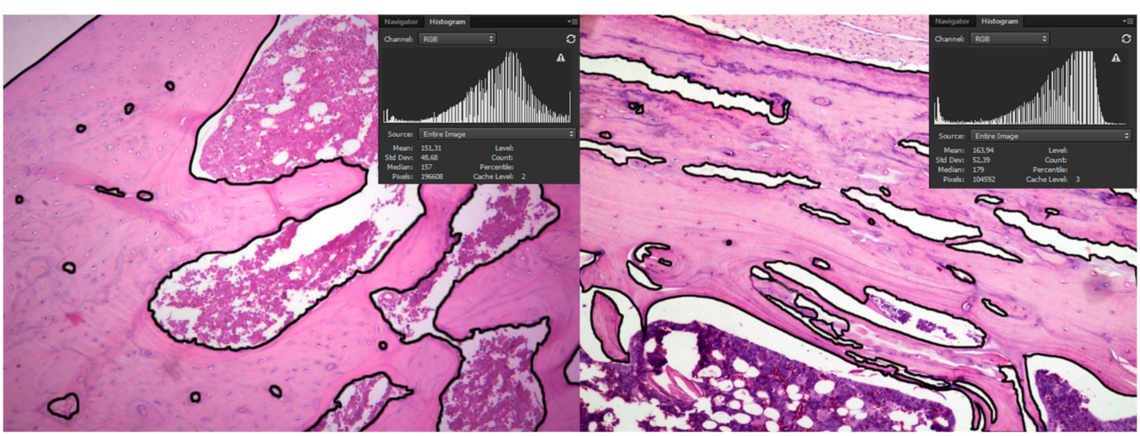

Fig. 2 Quantification of bone volume (BV) using Photoshop CC. **a** Mandibular histological section. **b** Tibial histological section

Table 1 Tomographic analysis. Bone density quantification, in Hounsfield units (HU)

Group	*N*	Mandible mean (SD)	Tibia mean (SD)	
Zoledronic acid (ZA)	12	1619.32 (99.66)	1569.67 (199.56)	$**p = 0.449$
Clodronate (CL)	12	1567.59 (143.54)	1598.81 (145.48)	$**p = 0.602$
Control	10	1550.54 (136.85)	1612.22 (238.65)	$**p = 0.487$
		$*p = 0.419$	$*p = 0.871$	

SD, standard deviation; *p*, *p* value

*ANOVA test, comparison between BP and control groups, for each bone

**Student's *t* test, comparison between different bones, for each drug group

Discussion

In this study, the comparison of mandibular cortical BD values between two different BPs and control, measured by CBCT, did not show a statistical difference. It was shown that cortical BD in BP-treated patients with MRONJ stages 0 to 3, compared to healthy patients, did not differ significantly when assessed with CT. In addition, the cortical bone width and cancellous BD were increased in the patients with MRONJ [22]. Individuals with MRONJ displayed decreased intracortical CBCT density, when compared to age-gender matched controls in the study by Gönen et al (2018), although cortical bone thickness was also increased [23]. The cancellous BD values were not significant. According to the authors, the results indicated that BP alters cortical bone metabolism, possibly by suppressing intracortical bone remodeling in the mandible [23, 24].

The comparison of BD values of a jaw and long bone did not show a significant difference in this study. Gong et al (2017) performed tooth extractions in the jaws and drilled hole defects in the ilium and tibia of rats that received ZA [18]. No difference in the BV fraction of jaw between the ZA group and controls was observed, using microCT. However, both BV fraction and cortical bone thickness were increased in the peripheral bones, compared to control. Using ELISA, these authors demonstrated a decreased RANKL/OPG ratio in the jaws and an increased ratio in peripheral bones. Altogether, this indicates that ZA strongly suppresses alveolar bone remodeling after tooth extraction, in conjunction with more active bone formation in the peripheral bones following injury [18].

Vermeer et al (2017) used microCT to evaluate the BV fraction and tissue mineral density (TMD) in jaws, tibiae, and femurs of mice not submitted to any surgical intervention, at three different times (1, 3, and 6 months) [25]. These authors observed that BV and TMD were significantly higher in the jaws compared to long bones. These results were similar for TMD at all time points of drug administration. The same authors demonstrated in vivo that jaw bone marrow cells internalized more BPs compared to long bones, suggesting bone site-specific responses to BPs [26].

The comparative results for CL in the present study, regarding long bone BD, were in accordance with the literature. Koivukangas et al. demonstrated that long-term treatment with CL, in both growing rats and adult rats, did not cause changes in BD values in the femur and tibia, as determined by peripheral quantitative CT [27, 28]. Moreover, resistance to fracture remained unchanged for the long bones, which was similar to the observations of other studies [27–29]. There are no data in the English language literature about the effects of CL in the mandible, which does not allow comparisons with this study.

Oyhanart et al. (2015) evaluated, histologically, the BV in the interradicular bone area of the mandible and tibia of growing rats that received alendronate, without any surgical intervention [2]. The authors observed increased BV in the experimental group, compared to control, in conjunction with a decrease in the total thickness of epiphyseal cartilage of tibia [2]. The histomorphometric measurement of mineral apposition rate in adult rats showed that ZA reduces bone formation in the jaws after 3 months of treatment, but not after 6 months. In long bones (tibia and femur), this reduction was observed

Table 2 Histomorphometric analysis. Proportion (%) of bone volume (BV)

Group	*N*	Mandible % (SD)	Tibia % (SD)	
Zoledronic acid (ZA)	12	66.92 (7.52)	59.41 (10.51)	$**p = 0.056$
Clodronate (CL)	12	66.27 (17.32)	55.93 (10.27)	$**p = 0.089$
Control	10	68.11 (8.98)	55.75 (14.25)	$**p = 0.032$
		$*p = 0.939$	$*p = 0.698$	

SD, standard deviation; *p*, *p* value

*ANOVA test, comparison between BP and control groups, for each bone

** Student's t test, comparison between different bones, for each drug group after 3 and 6 months of treatment [25]. The same study showed that long-term treatment with ZA reduced the number of jaw bone marrow cells, without affecting the number of long bone marrow cells. The results showed that N-BPs can affect long bone and jaw bone turnover differently in vivo.

Studies evaluating the effects of non-N-BP on jaws and long bones have shown similar results as ours here. Previously, the volume of cancellous bone and the mineral apposition rate of femur and tibia in rats were not altered by long-term treatment with CL, although the bone growth rate of femur decreased [27–29]. In addition, Vasconcelos et al (2012) quantified vital bone in the maxilla of adult rats that received CL, i.p. (at the same dose as in the present study), by means of histological analysis, without osseous surgical procedures [4]. The authors concluded that the proportion of vital bone was significantly greater in the control group compared to the CL group. Curiously, the present study found a statistically significant difference in the control group, with higher proportion in the jaw, compared to tibia. It is known that BPs promote nearly complete suppression of bone turnover, but in this study, we could not explain why a greater effect was observed in the control group, when compared with test groups.

Hence, we can infer that bone interventions and age of subject might alter osseous tissue response under N-BP therapy, which may also occur in a bone site-specific manner. The absence of any difference between groups and bones in the present study may be partially explained by (1) BP dose and administration period, (2) lack of different time point evaluations, and (3) use of adult animals. Although our BP administration regimen was sufficient to induce osteonecrosis [4, 30], the cumulative dose of ZA administered by Vermeer et al (2017) was considerably higher than ours (0.5 mg/kg weekly vs. 0.6 mg/kg monthly). Additionally, the same study evaluated different time points, showing the effect of time-related response. The differences in study design, including the stage of development of individuals, hamper suitable comparison between studies. Additionally, CL is the most prescribed non-N-BP, but there are few reports in the literature about the effects of this class of BF using animal models.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Informed consent This article does not contain any studies with human participants performed by any of the authors.

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