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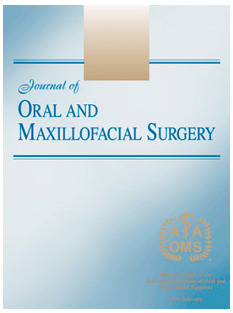

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Zoledronic acid induces site-specific structural changes and decreases vascular area in the alveolar bone

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Abstract

Purpose: The aim of this study was to assess the effect of a relevant regiment of zoledronic acid (ZA) treatment for the study of bisphosphonate-related osteonecrosis of the jaw (ONJ) on alveolar bone microstructure and vasculature. The subobjective was use three-dimensional imaging to describe site specific changes induced by ZA in the alveolar bone. **Methods:** Five Wistar rats received ZA (0.6 mg/kg) and five (controls) received saline solution in the same volume. The compounds were administrated intraperitoneally in five doses each 28 days. The rats were euthanized after 150 days of the therapy onset. Mandibles were scanned using a high-resolution (14μm) micro-computed tomography (Micro-CT); decalcified; cut into histological slices (5μm); and stained with Hematoxylin and Eosin. Bone quality parameters were calculated using CT-Analyser (Bruker, Belgium) in two different volumes of interest (VOI) (1.the region between the first molar roots and; 2.the periapical region under the first and second molars apex). Blood vessels density (VD) and bone histomorphometric parameters were only calculated for the region between the roots of the first molar using AxioVision Imaging 4.8 (Carl Zeiss, Germany). **Results:** ZA treated rats presented a significant increase in percentage of bone volume and density ($p<0.05$) with thicker and more connected trabeculae. Furthermore, the ZA group also presented a significant decrease in the size of the marrow spaces and nutritive canals and in the VD ($p<0.05$). In the Micro-CT evaluation, VOI-2 presented better outcomes in measuring ZA effect on alveolar bone. **Conclusion:** ZA treatment induced bone corticalization and decreased alveolar bone vascularization. VOI-2 should be preferred for Micro-CT evaluation of the effect of BP in the alveolar bone. The current analysis allows to characterize the effect of ZA on alveolar bone and its vascularization. Results of this analysis may add further knowledge to the understanding of ONJ physiopathology.

Key-words: Bisphosphonates; Bone Density Conservation Agents; Bisphosphonate-associated osteonecrosis of the jaw; micro-computed tomography; Cancellous Bone, Blood Vessels

Introduction

Bisphosphonates (BP) are widely used in the treatment of diseases characterized by increased bone reabsorption, such as osteoporosis and metastatic bone disease [1, 2]. In these diseases, the BP act mainly by suppressing osteoclast activity, by inducing apoptosis and, consequently, resulting in decreasing rates of bone remodeling [3, 4]. Although benefits of BP are well known, important late adverse effects mainly related to long-term and high dose therapies (i.e. atypical femoral fractures and osteonecrosis of the jaw; ONJ) have been reported [5-8]. The major concern regarding long-term use of BP is related to the possibility of excessive suppression of bone turnover that could impair bony strength and response to local injures [3, 7]. This concern is supported by evidence that long-term high-dose BP therapy compromises bony response to microdamage in animal models [9-12].

The medication-related ONJ lesion is characterized as an exposed bone that persists for more than eight weeks in patients treated with antiresorptive or antiangiogenic agents and with no prior radiation therapy or metastatic disease to the jaws [13]. The majority of BP-related ONJ cases are reported after intravenous administration of nitrogen containing BP (pamidronate and zoledronic acid; ZA) to treat skeletal malignancies [14]. The pathophysiology of BP-related ONJ still remains unclear. Several pieces of evidence support the notion that remodeling oversuppression and impaired vascularization induced by BP play a role in ONJ development [15-19].

Micro-CT imaging allows non-destructive direct quantification of cancellous bone structure and is widely used in the investigation of BP-induced changes in long bones in animal experiments [20-25]. However, few studies have investigated the effect of BP in the alveolar bone structure and the methodologies applied are variable [17, 26]. We hypothesize that the suppression of bone remodeling induced by BP treatment could

induce alveolar bone changes leading to a sclerotic and less vascularized bone, making the bone more vulnerable to ONJ lesion development.

Hence, the overall aim of this study was to investigate the effect of BP (zoledronic acid; ZA) treatment in a rat model, using a dedicated and standardized quantification method for site-specific characterization of alveolar bone structure and vascularization using Micro-CT scans and histology.

Materials and Method

Experimental animals

Ten Wistar albino male rats (*Rattus Norvegicus*), 12-weeks-old, weighing 271g ± 30g from the Central Animal Laboratory of the Bauru School of Dentistry, University of São Paulo were used in this study. The animals were housed in cages lined with sterile shavings. Cages were kept in a room with controlled air circulation (15-changes/hour), humidity (45-75%), temperature of 22ºC and artificial lighting with 12-hour light and 12-hour dark photoperiod. Solid food and water were available at libitum. Physical health and activity were checked daily. The experimental protocol was approved by the Animals Ethic Committee of Bauru School of Dentistry, University of São Paulo (protocol number 022/2014).

Experimental design

The medication regiment was performed according to the recommendation of Maahs and colleagues (2011) to induce ONJ in Wistar rats [27]. After two weeks of

acclimation, the rats were weighed, numbered and randomly divided into two groups with five animals: Experimental (ZA; 0.6 mg/kg Zometa, Novartis, São Paulo) and Control (CTR; 0.9% sodium chloride in the same volume of the experiential group). The solutions were administered intraperitoneally in five doses every 28 days. Body weight was recorded before the solution administration to determine the dose for each animal.

After 150 days from the treatment onset, the animals were sedated and euthanized with a solution of ketamine hydrochloride (Dopalen® - ketamine hydrochloride 10 mL and Nasedan® - xylazine 10 mL, CEVA, São Paulo, Brazil).

Micro-computerized tomography (Micro-CT)

The right hemimandibles were cut at the distal of the third molar and mesial of the first molar and placed in an Eppendorf tube of 1.5 ml embedded in saline solution and scanned using a high resolution micro-computerized tomography system Skyscan 1174® (Bruker, Kontich, Belgium). Scanning parameters were set at 50 kVp, 800 μA, frame averaging of 6 and 180° rotations with an angular step of 0.8°. A 0.5 mm aluminum filter was used to minimalize beam hardening effects and to reduce noise in the images. With the same settings hydroxyapatite phantoms of 0.25 g/cm³ and 0.75 g/cm³ were scanned to perform a bone mineral density (BMD) calibration with respect to the attenuation values. NRecon software® (version 1.6.5, Bruker, Belgium) was used for reconstruction of the raw images with isotropic voxel size of $14\mu m^3$.

Histology

Hemimandible segments were demineralized in 4.13%-EDTA (ethylenediamine tetraacetic acid - Titriplex III Merck®, Germany) aqueous solution containing 0.44% sodium hydroxide (LabSynth®, Diadema, Brazil) in pH 7.2 at room temperature, for approximately 40 days, with weekly changes of the solution. Subsequently, hemimandibles were subjected to histological processing and embedded in paraffin plus synthetic resin (Histosec, Merck KgaA, Germany). Eight longitudinal semi-serial 5-µm thick-sections with an interval of 100-µm between sections were obtained for each sample and stained with hematoxylin-eosin (HE).

Bone morphometric analysis

For micro-CT morphometric analysis, after image reconstruction, images were manually registered with the histological sections to ensure the evaluation of the same region in both techniques. Morphometric parameters were calculated in CT-analyzer software® (Bruker, Belgium). As mandibular alveolar bone structure around tooth roots showed more compact than the basal bone situated apically, it was decided to allow for site-specific analysis. In order to do so, two different adaptive volumes of interest (VOI) were selected to analyze these regions separately: (VOI-1) including the alveolar bone between the mesial and distal roots of the first molar; and (VOI-2) including the cancellous alveolar bone in the region of molars apex and excluding other anatomical regions (i.e.: mandibular canal, cortical bone, periodontal ligament and teeth; figure 1). The VOI-2 size was the same for all samples. The structures within the VOI-1 and VOI-2 were segmented using a global automatic threshold algorithm. For VOI-1 just the morphometric parameters that could also be evaluated using histomorphometry were

calculated: bone volume fraction (BV/TV;%), bone surface density (BS/TV;mm^2/mm^3), trabecular thickness (Tb.Th; mm), trabecular number (Tb.N; 1/mm) and trabecular separation (Tb.Sp; mm) [28]. For VOI-2, morphometric indices were calculated and grouped according to terms clinically used for bone quality evaluation [29]: (1) Bone quantity: BV/TV, BS/TV and Tb.Th; (2) Bone structure: Tb.N, Tb.Sp, connectivity density (Conn.Dn; $1/mm^3$), total porosity percentage (Po[tot]; %), trabecular pattern factor (Tb.Pf;1/mm), structure model index (SMI) as well as (3) bone density: (BMD).

For histomorphometric analysis, eight sections of each sample were selected. Digital microscopic images with 5x magnification factor were obtained using a Axiocam HRc digital camera (Carl Zeiss, Gottingen, Germany) coupled to an optical microscope Axioskop 2® (Carl Zeiss, Gottingen, Germany), which had been previous calibrated. Measurements of Total Area (A_t), Bone Area (A_b) and Bone Perimeter Length (B_p) were performed using AxioVision imaging software (Carl Zeiss). Alveolar bone between the roots of the inferior first molar was evaluated. Morphometric parameters (BV/TV, BS/TV, Tb.Th, Tb.N and Tb.S) were calculated from the primary two-dimensional area and perimeter length measurements using the stereological method proposed by Parffit and colleagues (1983) following the SBMR nomenclature [28, 30].

Blood vessels assessed using histomorphometric analysis

In the same eight histological sections, the blood vessels in the alveolar bone between mesial and distal roots at the level of cervical and middle thirds were quantified using 20X magnification. This field was selected to exclude the region of the roots apex in order to avoid any change in vasculature induced by possible odontogenic

inflammation. The total area (TA) and the blood vessels area (VA) were manually delimitated and automatic calculated using AxioVision Imaging 4.8 (Carl Zeiss). The relative area of blood vessels was expressed as a percentage to the total area: (VA/TA) x 100.

Statistical Analysis

Statistical tests were conducted in IBM SPSS version 22.0 (IBM, New York, USA) and statistical significance of α=0.05 was admitted. The Shapiro-Wilk test was carried to access data normality. One-way MANOVA was used to estimate the effect of ZA treatment in the alveolar bone and to compare the changes between the experimental and control group. Linear models were used to aggregate variables into bone quantity (BV/TV, BS/TV, Tb.Th) and structure (Tb.N, Tb.Sp, Conn.Dn, Po[tot], Tb.Pf, SMI). Spearman correlation test was used to evaluate the relationship between VA/TA and BV/TV. Quantitative data of the Micro-CT and histomorphometric parameters are presented as means and SD in graphs.

Results

All animals showed good tolerance to the experimental conditions. Clinically, none of the rats presented oral lesions at the time of the euthanasia. Due to the curved anatomy of the first molar roots, in one control sample it was not possible to select the region of interest for histomorphometric analysis. Hence, that specific sample was excluded.

Assessment at the level of the alveolar bone (VOI-1)

The micro-CT morphometric calculation in the region including the alveolar bone between the mesial and distal roots of the first molar showed that ZA treated animals presented significant increased BV/TV ($p<0.001$) and thicker trabeculae (↑Tb.Th; $p<0.05$) with smaller marrow space (↓Tb.Sp $p<0.05$; figure 2).

The histological findings were consistent with the Micro-CT observations in VOI-1 and an increase of the bone area together with decrease in size of the nutritive canals and marrow areas was observed in the ZA group. Specimens obtained from the ZA group were also characterized by an increased osteoid deposition with prominent reversal lines. The presence of connective tissue inside the nutritive canals and congested blood vessels was also noticed in the ZA group (Figure 3). In the present experiment, necrotic bone sites could not be observed in any of the animals.

Stereological calculation, revealed that ZA treated rats presented significant increase in BV/TV ($p<0.001$) and significant decrease in Tb.Sp ($p<0.01$). Other bone morphometric parameters did not show any significant differences for ZA treated vs normal rats (Figure 2). Quantification of the area of blood vessels revealed that ZA treatment induced a significant reduction in the VA/TA ($p< 0.01$; figure 2). A strong negative correlation was observed between VA/TA and the BV/TV values obtained both in Micro-CT ($r= -885$; $p< 0.01$) and histomorphometry ($r= -0,898$; $p<0.01$), suggesting an increasing BV/TV along with a VA/TA decrease.

Assessment in the periapical region (VOI-2)

Regarding the micro-CT morphometric analysis in the periapical region under the first and second molars apex, ZA treatment presented a significant impact on alveolar cancellous bone mineral density ($F= 17.45$; $p<0.001$; $\eta^2_{p=} 0.68$) and quantity ($F= 24.78$; $p< 0.001$; $\eta^2_{p=} 0.75$). A significant increase the BV/TV ($p<0.001$) with thicker (↑Tb.Th ($p<0.001$) and less complex trabecular structure (↓BS/TV; $p<0.05$) was noticed in the ZA group compared to the control. Significant impact of ZA on cancellous bone structure was also observed ($F= 8.10$; $p< 0.001$; $n^2_{p=} 0.53$). In the ZA group, alveolar bone presented a plate-like structure (↓SMI; $p<0.01$) with more compact (↓Tb.N; $p<0.05$) and more connected (↓Tb.Pf; $p<0.001$ and ↑Conn.Dn; $p<0.01$) trabeculae. Significant reduction in marrow spaces (↓Tb.Sp; $p<0.001$) and decreased bone porosity (↓Po[tot]; $p<0.001$) in relation to the control group was also noticed. These results are summarized in figure 4.

Discussion

In this study, the effect of a relevant regiment of ZA treatment for the study of bisphosphonate-related ONJ on jaw bone microstructure and vasculature of Wistar rats was established. The analysis method allowed to find marked differences for the ZA treated rats as compared to the controls. ZA treated rats presented markedly denser cancellous bone, with less complex structure, thicker and more connected trabecula. As a consequence of this sclerotic architecture induced by ZA, smaller marrow spaces and nutritive canals, leading to significant reduction in the percentage of blood vessels area were observed. Together, these findings support the hypothesis that bone turnover

oversuppression induced by BP can induce bone corticalization and seriously impact vascularization. This impairment may play a key role in hampered jaw bone healing and further development of ONJ [15, 31, 32].

In agreement with the present findings, previous studies demonstrated that ZA increased jaw bone density [17, 26]. Nevertheless, these studies evaluated bone volume in post-extraction alveolar socket of ONJ models, where the healing processes, inflammation and disease itself might influence the results [17, 26] In the present investigation, a medication regiment capable of inducing a high rate of ONJ when combined with tooth removal was reproduced [27]. However, local trauma was not induced. In this way, we described alveolar bone structure changes induced by ZA in healthy animals that might potentially constitute the first step in ONJ development.

Manifest bone remodeling suppression induced by long-term BP treatment has been previously associated with spontaneous bone matrix necrosis in the jaw of beagle dogs [33]. However, no such necrotic tissue was observed in our sample. This outcome may be primarily related to differences in bony tissue response among species, yet also to variations in the experimental protocol [34].

In the present study, morphometric parameters that reflect cancellous bone quality were objectively quantified in two different regions of interest. The first region was situated between the roots of the first molar as it was the closest region to the tooth roots (VOI-1), where masticatory forces can induce different bone remodeling rates from other regions of the jaw [33, 35]. Both micro-CT and histomorphometric evaluations showed a significant ($p<0.05$) reduction in size of marrow spaces and nutritive canals, and increase in bone quantity in ZA treated rats. These findings were accompanied by numerous thicker reversal lines in the ZA group, which can be associated with a higher rate of woven bone deposition and compromised reabsorption

caused–by ZA [32]. This is consistent with a reversal line pattern reported on ONJ patient's biopsies [32].

As a result of nutritive canals obliteration, a significant reduction in the percentage of blood vessels area was present in our sample. Furthermore, congested blood vessels were noticed in ZA group but not in controls, being indicative of less blood perfusion at the time of formalin samples fixation. In accordance with our findings, other investigations reported that ZA decreases blood vessels area, branching and connectivity in the jaw bone [18, 19]. Pamidronate, has also been shown to reduce blood flow in the tibia and femur in rats [36]. The reduction in blood flow and in vascular area could interfere with the ability of bone to respond to trauma and infection and lead to ONJ.

A comprehensive micro-CT analysis of the region under the apex of the first and second molars (VOI-2) indicated that the ZA treated jaw bone was characterized by an increased bone quantity, less complex trabecular structure, a more plate-like pattern, increased connectivity, smaller marrow spaces and increased BMD. These changes were confirmed by histological analysis and were more prominent compared to the alveolar bone between the molar roots (VOI-1). This raises the hypothesis that, like observed in osteoporotic rat models, BP-induced structural changes detected by micro-CT are site-specific in the mandible and vary according to the volume of interest selected for evaluation [35, 37]. Site-specific variation of bone structure, may explain why in the interradicular region with inherently dense bone available, further deposition of mineral content together with impaired bone remodeling induces the diminution of the marrow spaces. Yet, in the periapical area, bone is more trabecularized and less dense, inducing a morphology with fewer, plate-like and thicker trabeculae [38].

Micro-CT allows the direct tridimensional investigation of the trabecular structure with high reliability when compared with the standard histomorphometric measurements [20, 39]. However, a limitation of this method lies in the inability to access cellular changes and the need of contrast in the investigation of vasculature [20, 39]. Hence, a complementary histologic analysis was performed. Most investigations directly access micro-CT and histology without an additional image processing to minimize possible discrepancies induced by the selection of different regions of interest in each method [20]. In the present investigation, a spatial alignment between micro-CT images and histological couples was performed. Generally, micro-CT overestimates morphometric parameters when compared to histomorphometric evaluation. This is probably the result of an additional segmentation step used in micro-CT [40]. The discrepancy between voxel size (14µm) and histological slides thickness (5µm) should be encountered as this may have contributed to micro-CT overestimation, thus being a limitation of such approach [20].

The present study was focused on the cross-sectional ex vivo micro-CT and histological evaluation, in this way no baseline was considered. An in vivo follow up that could cross-correlate both vascular and bony changes induced by BP in the jaw bone should be encouraged. Furthermore, the techniques here applied are only able to measure tissue morphology. Since bone blood supply is influenced both by vessel morphology and function, future investigations should combine methods able to address these both features [41].

In conclusion, BP treatment in a relevant dose for the study of ONJ in Wistar rats is able to induce corticalization and decrease vascularization in the jaw. Impairment of bone vascularization, in presence of local trauma and infection, could hamper inflammatory response and healing capacity and consequently, make the jaw bone more

vulnerable to ONJ development [15]. The dedicated site-specific bony assessment allows concluding that future studies on ONJ in wistar rats might need to consider micro-CT analysis of bony areas in the periapical molar region. This study offers a unique insight into changes in alveolar bone and its vascularization induced by BP, thus contributing to our understanding on the potential induction of ONJ. Further studies should consider applying such dedicated analysis of bone morphometric parameters to allow predicting alveolar bone changes being indicative for increased ONJ risk in patients under BP therapy.

Conflict of interest

The authors declare no conflict of interest

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Figure 1: Cancellous bone segmented within the volumes of interest selected for the 3D morphometric analysis; VOI-1 (green) VOI-2 (red).

Figure 2: ZA treatment induced corticalization of the alveolar bone in the region between the roots of the first molar. Smaller marrow spaces were observed in the ZA

group (c and d) in comparison to the control group (a and b). Significant increase in bone quantity (BV/TV) and decrease in Tb.Sp were observed in 3D Micro-CT (e) and histological (f) investigation. A decrease in bone vasculature was also observed in histological quantification (f). (asterisks: $p<0.05$).

Figure 3: a) Control: presence of larger blood vessels and nutritive canals and thin discreet reversal lines (arrows heads); b) ZA: presence of smaller nutritive canals with more connective tissue within and blood vessels (arrows), congested blood vessels (asterisks) and thicker basophilic irregular reversal lines (arrows heads). 20X magnification.

Figure 4: Significant changes were induced by ZA treatment in bone density, quantity and structure in VOI-2 (Asterisks: $p>0.05$).