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Long-term effects of Zoledronic acid on alveolar bone remodeling and quality in the jaw of an oncological rat model

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Abstract

Despite great interest in bisphosphonate-induced osteonecrosis of the jaw (ONJ), the underlying pathology remains a matter of debate. In most reports, the etiology of ONJ seems to be ascribed to bisphosphonate-related over suppression of bone remodeling. While suppression of bone remodeling is indeed the prime action mechanism of bisphosphonates, the destructive ONJ effects at the level of the jaw bone are ignored or minimalized when prescribing these drugs. The present study therefore investigated the long-term effects of oncological Zoledronic Acid (ZA) doses on the three factors which may comprise jaw bone:(1) bone quantity, (2) bone structure and (3) bone mineral density (BMD). Twenty-four, healthy, Wistar rats were randomly divided in an experimental group (n = 12) receiving 0.6 mg/kg body weight/month ZA and a control group (n = 12) receiving an equal volume of saline for 5 consecutive months. The ZA doses were equivalent to the doses given to oncologic patients. After animal sacrifice, their mandibles were ex vivo scanned using 1174 micro-CT (Bruker) and the obtained scans were spatially aligned. One mean volume of interest containing only trabecular bone below the apex of the molars was selected, in which 3D quantitative morphometry and BMD were calculated using CT-Analyser (Bruker). Structural-related parameters showed significantly ($P < 0.001$) increased osteoslerotic characteristics of the alveolar bone (↑ Tb.Pf; ↓ Tb.Sp; ↓Tb.N; ↓ SMI; ↓ Conn.Dn) in ZA-treated rats as compared to the controls. These findings were confirmed by a significantly ($P < 0.001$) increased bone quantity (↑ BV/TV; ↑ Tb.Th; ↓ BS/TV; ↓ Po[tot]). Finally, the alveolar bone displayed a significantly ($P < 0.001$) higher BMD after prolonged ZA therapy. These results are strongly supportive for bisphosphonate-induced oversuppression of alveolar bone remodeling. The bone becomes more sclerotic leading to less vascularization and an ONJ prone jaw.

Keywords: *bisphosphonates, zoledronic acid, bone remodeling, micro-ct, bone quality, bone density, quantitative bone morphometry, biphosphonate-induced osteonecrosis of the jaw*

Introduction

Bisphosphonates are widely and effectively used for treatment and prevention of osteoporosis, and other diseases characterized by increased bone resorption such as symptomatic skeletal events associated with solid malign tumor metastases in prostate, lung and breast cancer [1]. Bisphosphonates have a selective and high affinity for bone tissue, especially in areas of high bone remodeling, where they slow down osteoclast-mediated bone resorption and prevent osteoblast apoptosis resulting in lower rate of bone turnover [2]. Compared with treatment schemes used for osteoporosis, treatment of bone metastases requires use of bisphosphonates with higher efficacy (e.g. Pamidronic or Zoledronic Acid) given at much shorter dosing intervals [3]. Resulting in many fold higher exposed doses for oncologic patients, which makes them more susceptible for adverse side effects. The most reported adverse effect of long-term bisphosphonate treatment is the osteonecrosis of the jaw (ONJ) with an overall prevalence of 1 to 15 % in patients with cancer [4]. ONJ is clinically defined as an area of exposed bone in the maxilla facial region that has persisted for more than eight weeks in patients who have taken or are currently taking BP, while having no history of radiation therapy to the craniofacial region [4]. Despite great interest in bisphosphonate-induced ONJ, there are still many hypothesis concerning its underlying pathology [5]. The primary cause that is identified in most reports or reviews on ONJ is the bisphosphonate-induced oversuppresion of bone remodeling [6]. Recent animal studies have shown the greatest oversuppression in the alveolar bone of the mandible [7-9]. These osteosclerotic changes in the jawbone microenvironment may lead to destruction of the residing blood vessels [10-12] and accumulation of microfractures [13-15]. Without the supply of necessary nutrients, bone will start to resorb, resulting in severe necrosis of the jaw [16]. Inflammation after tooth extraction or surgical trauma due to the large concentration of bacteria in the mouth or dental diseases may accelerate and aggravate the bone resorption process [17]. While suppression of bone remodeling is the principal mechanism of action of bisphosphonates, its effects on the alveolar bone quality are largely neglected [18]. From literature, it is often not easy to determine what bone quality represents, as it is not only a matter of

bone mineral density (BMD), but also encompasses other factors such as bone quantity and morphology [19]. Micro-Computed Tomography (micro-CT) has been validated and considered the gold standard to evaluate all three-dimensional (3D) aspects of trabecular bone quality [20]. The present study aimed to evaluate the long-term effects of Zoledronic Acid (ZA) on the alveolar bone quality in an oncologic dose rat model using micro-CT. Secondly, a novel image analysis procedure was introduced to objectively compare the alveolar bone quality by means of 3D morphometric parameters and BMD measurement's.

Materials and Methods

Animal care and bisphosphonates administration

Animals were handled according to the study protocol reviewed and approved by the ethical committee of the Bauru School of Dentistry, University of São Paulo, Brazil (reference number: 03/2013). Twenty-four, male, 12-weeks old Wistar rats (Central Animal Laboratory of Bauru Dental School, University of São Paulo, Brazil) with an average body weight of approximately 300g were randomized into two groups: one experimental group (n = 12) and one control group (n = 12). The experimental group received 0.6 mg/kg Zoledronic Acid (Zometa, Novartis Pharma AG, Basel, Switzerland) treatment throughout the 20-week experiment. ZA was administered by intraperitoneal injection every 28 days with a total of 5 doses. Body weights were recorded at time of drug administration in order to recalculate the solution dose. The ZA dose was based on Maahs et al. (2011) and was therefore considered equivalent to the human dose level given to oncologic patients adjusted for rats' weight, metabolic rates and treatment period [21]. The control group received an equal volume of saline following the same administration scheme. Animals were euthanized 150 days after initiation of treatment with an anesthetic drug cocktail (Dopalen - Ketamine Hydrochloride 10 ml and Anasedan - Xylazine 10 ml , CEVA, São Paulo, Brazil). The mandible was disjointed from the maxilla, defleshed and stored in formaldehyde 10% after micro-CT scanning.

Micro-CT scanning

The hemimandibles were cut at the distal end of the third molar and mesial end of the first molars in order to fit in the field of view of the high-resolution micro-CT scan protocol. Each sample was placed in a 1.5 ml Eppendorf tube embedded in saline solution and scanned with a SkyScan 1174 micro-CT (Bruker, Kontich, Belgium). Scanning parameters were set at 50 kVp, 800 µA, frame averaging of 6 and 180° rotation with an angular step of 0.8°. With the same settings BMD phantoms of 0.25 g/cm³ and 0.75 g/cm³ were scanned to perform a BMD calibration with respect to the attenuation values. A 1 mm aluminum filter was used to minimalize beam hardening effects that may potentially affect the BMD measurements and to reduce noise in the images. After scanning, the raw image stack was reconstructed in NRecon software(version 1.6.5, Bruker micro-CT) with isotropic voxel size of 14 µm³.

Bone quality evaluation

All micro-CT scans underwent the same image processing workflow shown in figure 1. To ensure a standardized comparison of trabecular bone structures between the control and ZA-treated group, it was opted to use the same volume of interest (VOI) for the entire dataset. All micro-CT images were therefore spatially aligned in the same coordinate system using a rigid transformation based on mutual information [22]. After precise registration, one averaged VOI was selected that only consists of trabecular bone below the apex of the molars in all the samples. Nearby anatomical regions that could influence the results were excluded e.g. mandibular canal, cortical bone, periodontal ligament and the incisor tooth. After calibration, an average BMD was calculated within the VOI. The bone structures within the VOI were segmented using an automatic adaptive threshold algorithm in CT-Analyser (Bruker, Kontich, Belgium). The validity of the segmentation result was visually confirmed by overlaying the segmented network on the original image. Quantitative 3D bone morphometry was performed to objectively compare differences in bone quantity and structural network between control and ZA-treated groups. Based on the final binary images, 3D morphometric parameters were automatically calculated in CT-Analyser and named according to the Parfitt system [23]. The morphometric indices were divided in regularly used terms in clinical bone quality evaluation [24]. (1) Bone quantity relates to the amount of bone in a selected VOI and includes bone volume fraction (BV/TV; %), bone surface density (BS/TV; mm²/mm³), trabecular thickness (Tb.Th; mm). (2) Bone structure is associated with the trabeculae microarchitecture and includes trabecular separation (Tb.Sp; mm), connectivity density (Conn.Dn; 1/mm³), total porosity percentage (Po[tot]; %), trabecular pattern factor (Tb.Pf; 1/mm), structure model index (SMI), trabecular number (Tb.N; 1/mm).

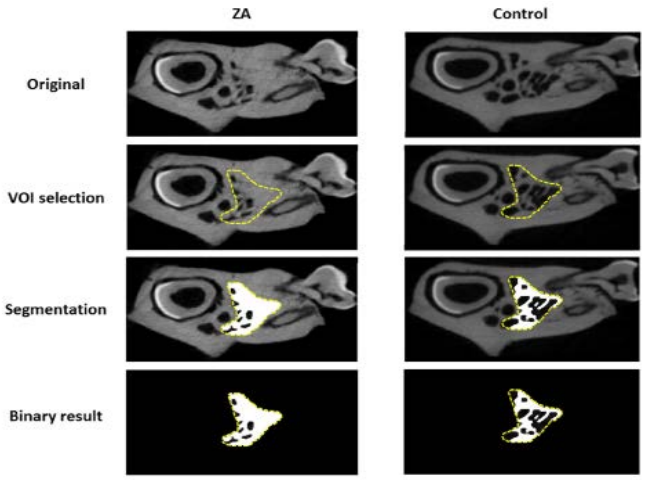

Figure 1. *Image processing steps on micro-CT scans of Zoledronic Acid (ZA)-treated and control rat mandibles. All micro-CT images were spatially matched in the same coordinate system. After accurate registration, one mean volume of interest (VOI) that only contains alveolar bone below the apex of the molars was calculated and used in all the samples. Trabecular bone structures were segmented using an adaptive threshold. Based on the resulting binary images, 3D morphometric indices were automatically calculated and 3D models were made.*

Statistical analysis

The number of rats was calculated using the BV/TV values of a previous study that compared control and high-dose ZA-treated rats [25]. A power analysis in G*Power 3.1 suggested a sample size of 9 rats per group assuming 95% power with α of 0.05 [26]. The sample was increased to 12 rats to anticipate potential loss of animals during the experiment. Further statistical analysis was conducted in IBM SPSS statistical software (Version 22.0, IBM, New York, USA). The significance level α was set for all statistical

tests at 0.05. A one-way MANOVA was performed to control for repeated measurements in the same rat mandible. Aggregate variables were created for the 3 factors that comprise bone quality: (1) bone quantity (BV/TV, BS/TV, Tb.Th), (2) bone structure (Tb.N, Conn.Dn, Tb.Pf, SMI, Tb.Sp, Po[tot]) and (3) BMD to examine differences between the control and ZA-treated group.

Results

General observations

Initial boy weight was the same among control (304 ± 17g) and ZA (301 ± 15g) groups. All animals tolerated the treatment well.

Bone structure

Quantitative 3D morphometry of micro-CT images revealed that long-term oncologic ZA treatment had a significant ($F = 6.10$; $p = 0.001$; $\eta_p^2 = 0.68$) impact on the alveolar bone structure. ZA-treated rats showed strong osteoslecrotic characteristics in the trabecular network compared to the control rats (Figure 2). This was mainly due to significant ($p < 0.001$) decrease in trabecular space (8% ↓Tb. Sp; 36% ↓ Po[tot]), lower amount of trabeculae (35% ↓ Tb.N) with a more plate-like pattern (11% ↓ SMI) (Figure 3). What resulted in a significantly increased connectivity (43% ↑ Conn.Dn; 34% ↓Tb. Pf) (Figure 3).

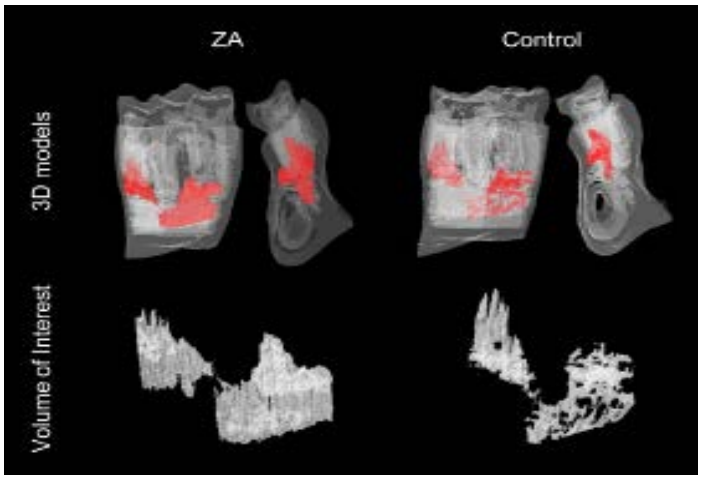

Figure 2. Visual comparison of the alveolar bone region (red) shows a higher bone quantity and osteoslerotic bone structure in rats that were under oncologic Zoledronic Acid (ZA) treatment compared to controls

Bone quantity

Along with drastic structural alterations of the alveolar bone, one can visually observe a higher bone quantity in the selected VOI (Figure 2). This was confirmed significant changes ($F = 14.88$; $p < 0.001$; $\eta_p^2 = 0.69$) in bone quantity-related morphometric indices after ZA usage. Compared with the control group, alveolar bone was significantly ($p < 0.001$) larger in quantity (36% ↑ BV/TV), explained by thicker (49% ↑ Tb.Th) and less complex trabeculae (39% ↓ BS/TV) (Figure 3).

Bone density

The alveolar bone showed a significantly ($F = 19.88$; $p < 0.001$; $\eta_p^2 = 0.78$) higher BMD after long-term ZA therapy compared to control rats, associated withan 82% increase in alveolar bone density (Figure 3).

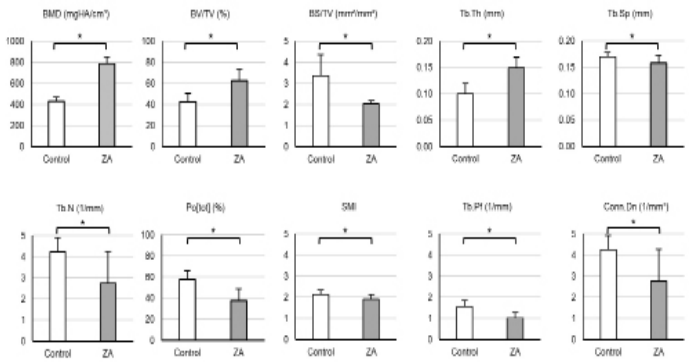

Figure 3. *Long-term oncologic Zoledronic Acid treatment (grey bar) had a severe impact on the alveolar bone remodeling and quality in the rat mandible. This was emphasized by significant (*) differences in bone mineral density (BMD) measurements, bone quantity-related (bone volume fraction [BV/TV], bone surface density [BS/TV], trabecular thickness [Tb.Th], total porosity [Po[tot]]) and structural-related (trabecular separation [Tb.Sp], trabecular number [Tb.N], structural model index [SMI], trabecular pattern factor [Tb.Pf], connectivity density [Conn.Dn]) morphometric indices. Y-axes display the quantitative values of the various indices expressed. Error bars display the standard error. Asterisks (*) indicate $P < 0.001$.*

Discussion

The present research investigated the long-term effects of oncological Zoledronic Acid (ZA) doses on alveolar bone quality in an oncological rat model by applying quantitative 3D morphometry and bone mineral density (BMD) measurements on micro-CT scans. The results are strongly supportive for bisphosphonate-induced oversupression of alveolar bone remodeling in three ways. First, structural-related parameters showed increased osteoslerotic characteristics of the alveolar bone in ZA-treated rats compared to the control group. Secondly, these findings were confirmed by a significantly increased bone quantity in the selected VOI. Thirdly, the alveolar bone displayed a significantly higher BMD after prolonged ZA therapy. These results are in line with the general hypothesis that bone quantity and BMD increase when the remodeling rate decreases [26].

Previous micro-CT studies have demonstrated a similar increase in bone quantity after prolonged use of oncologic bisphosphonate doses [17,25,28-32]. Most of these studies considered bone volume (BV) or the normalized bone volume fraction (BV/TV) as primary morphometric parameter for evaluating bone quality. However, if the remodeling rate is locally inhibited then this would first manifest itself in thicker trabeculae (↑ Tb.Th and ↓ BS/TV), which finally would result in an increased bone quantity (↑ BV and ↑ BV/TV). This effect should be accompanied by a long-term effect on the alveolar bone microarchitecture and BMD [12,28,32,33]. Analysis of these additional factors of bone quality are largely neglected. In this study, the alveolar bone showed a significant ($P < 0.05$) alteration towards a typical osteoslerotic bone structure in the ZA group characterized by less trabeculae (↓ Tb.N) with plate-like pattern (↓ SMI), high trabecular interconnectivity (↑ Conn.Dn, ↓ Tb.Pf), small trabecular spaces (↓ Tb.Sp, ↓ Po[tot]) and increased BMD.

It is important to emphasize that the applied image processing has a significant impact on the reliability of the morphometric results. In contrast to previous micro-CT studies, 3D morphometric indices and BMD values were calculated in the same VOI for the entire dataset. This was made possible by a spatial alignment of all rat mandibles in the same coordinate system prior to analysis. Since we were specifically interested in examining the alveolar bone remodeling, only the trabecular bone below the apex of the molars was selected and nearby anatomical structures that could influence the results were excluded. Therefore, an irregular adaptive VOI was used in contrast to the standard rectangular or circular contour that is most commonly used in micro-CT bone evaluations. This new analysis procedure allows a standardized and objective comparison of the alveolar bone quality between groups. The segmentation process was optimized by choosing an adaptive thresholding method, as it showed to give the best segmentation results for trabecular bone structures [34].

Most animal studies that investigate the pathophysiological mechanism of bisphosphonate-induced ONJ use animal models that are systemically treated with high-dose bisphosphonates combined witha specific risk factor for ONJ development. These are typically tooth extractions and/or induction of severe dental diseases [17,25,30,31]. Recently, there has been increasing criticism on these ONJ animal models, as they not fully represent the clinical reality [17]. First, tooth extractions are strong contraindications in oncology patients. Secondly, dental disease rarely is the sole cause of the development of ONJ while using high-dose bisphosphonates, which is still a non-healing socket after tooth extraction. In the present study, we specifically wanted to investigate in a preclinical model the hypothesis that cancer patients have a greater remodeling suppression within the jaw during oncologic treatment schedules. Therefore, we used a healthy animal model that mimics the oncological situation by using a bisphosphonate with higher potency (Zoledronic Acid) given long-term at frequent oncologic dosing schedule. In clinical practice, however, ZA is generally administered by intravenous route, while in current study the animals were treated by intraperitoneal injection due to its ease and safety management. Intraperitoneal administration results a lower effective dose than intravenous route injection [35]. Therefore, the intraperitoneal administered drug dosage was based on Maahs et al. (2011), which was adjusted to the therapeutic dose prescribed for cancer patients according to the rats weight, metabolic rates and treatment period [21].

When the jawbone becomes osteoslerotic, a decreased vascularization and accumulation of microfractures can be assumed, which may lead to an increased risk of ONJ [10-15]. Although these effects were assessed indirectly by evaluating the alveolar bone quality, future research is needed to quantify the amount of blood vessels and microfractures linked with osteoslerotic bone formation during long-term oncologic bisphosphonatedoses [12]. Current research was limited to a high-resolution ex vivo evaluation of the alveolar bone. Consequently, no baseline scan prior to the bisphosphonate intake could be taken in order to calculate percentage changes in bone quantity, quality and mineral density over time. In vivo micro-CT scans can provide more insight on the time course of the osteoslerotic bone formation.

The present study indicates the clinical significance of detailed radiographic 3D assessment of alveolar bone quality prior to surgical dental interventions in patients under bisphosphonate treatment. According to the most recent International Consensus paper, a high-resolution Cone-Beam CT or multi-slice CT are recommended in patients for whom ONJ is a clinical concern and teeth extractions are considered [4]. Recent studies have validated Cone-Beam CT and multi-slice CT for standardized alveolar bone quality evolution, opening perspectives for future clinical assessment [36,37].

In conclusion, the current results are strongly supportive for bisphosphonate-induced oversupression of alveolar bone remodeling. A standardized analysis procedure was used to objectively evaluate the alveolar bone quality by means of 3D morphometric parameters and BMD measurement's. The alveolar bone became sclerotic after prolonged high-dose ZA treatment characterized significantly increased BMD and bone quantity parameters, and altered alveolar bone structure compared to the control group.

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