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Early bony changes associated with bisphosphonate-related osteonecrosis of the jaws in rats: A longitudinal *in vivo* study

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ABSTRACT

Objective: To evaluate early bony changes in an animal model of Medication-Related Osteonecrosis of the Jaw (MRONJ) at the side of the local trauma and at the contralateral side, comparing with a control group. Bony changes were evaluated by Microcomputed Tomography (MicroCT) at three times points: at baseline (T0), after drug administration (T1) and after dental extraction (T2).

Design: Two groups were compared: the experimental group in which zoledronic acid (ZA) was administered (17 rats) and the control group (13 rats). Dental extractions of the lower left first molars were performed in all animals. The left side was considered as the supposed affected area in the ZA group, and the right side was considered as the unaffected area. In these areas, the following structural microtomographic bone parameters were calculated: Bone Mineral Density (BMD), Trabecular Thickness (Tb.Th), and Bone Volume Proportion (BV/TV). The comparison of quantitative bone parameters among the different sides and experimental phases of both studied groups were performed by ANOVA-factorial.

Results: None of the animals of the control group developed MRONJ. In the ZA group, 76% presented bone exposure. From T0 to T1, Tb.Th and BV/TV increased, and in T2, the mean values were higher in ZA group than in the control group. BMD increased throughout the different phases of both groups.

Conclusions: Structural bony changes occurred in the ZA group at both mandibular sides before the dental extraction (T1). Tb.Th and BV/TV should be further investigated as potential early bone markers of MRONJ.

1. Introduction

Bisphosphonates (BPs) are antiresorptive agents whose actions interfere in bone remodeling mechanisms. These drugs bind to bone's hydroxyapatite and suppress osteoclast activity, reducing bone loss. BPs are widely used to treat several diseases which involve bone resorption such as osteoporosis, bone metastases, multiple myeloma, Paget's disease (Cremers & Papapoulos, 2011; Drake, Clarke, & Khosla, 2008). The most important side effect related to BP therapy is osteonecrosis of the jaws, firstly described in 2003 (Marx, 2003; Ruggiero et al., 2014). In the present days, the disease is known as Medication-Related Osteonecrosis of the Jaws – MRONJ (Ruggiero et al., 2014).

MRONJ was defined as exposed or necrotic bone in the maxillofacial region for at least 8 weeks in patients receiving anti-resorptive or anti-angiogenic medications, without radiation therapy to the jaws or obvious metastatic disease to the jaws. Bone remodeling or over-suppression of bone resorption, angiogenesis inhibition, constant microtrauma, suppression of innate or acquired immunity, vitamin D deficiency, soft tissue toxicity, and inflammation or infection were proposed to explain the still uncertain pathophysiology of the disease (Ruggiero et al., 2014).

Therapy time, medication type, local trauma, local bone response,

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genetic factors were also pointed out as risk factors related to the main etiological factors (Barasch et al., 2011; Rasmusson & Abtahi, 2014; Saad et al., 2012). Nitrogen-containing BPs's are the predominant cause of MRONJ, especially if these are administered intravenously. Zoledronic acid (ZA) is the most potent nitrogen-containing BPs, being associated to the highest risk for the disease (Cremers & Papapoulos, 2011; Mücke, Krestan, Mitchell, Kirschke, & Wutzl, 2016).

Signals and symptons for MRONJ are pain, dental mobility, swelling in oral mucosa, soreness, and bone exposure (Rasmusson & Abtahi, 2014). Image findings are nonspecific and include bone sclerosis, osteolytic changes, bone sequestrum, periosteal reaction and fracture of the jaws. Computed tomography (CT) is the more sensible imaging method for evaluating bony changes, providing better image resolution (Arce, Assael, Weissman, & Markiewicz, 2009; Chiandussi et al., 2006; Treister, Friedland, & Woo, 2010).

As the diagnosis of MRONJ is primarily based on clinical features, the early detection of the risk of developing an osteonecrosis in the maxillofacial area before showing exposed bone is still a challenge. Some imaging tools may aid in the identification of early bony changes (Taniguchi et al., 2016). Early detection of MRONJ and the ability to predict the areas where MRONJ might arise are important for the effective management of these patients.

Some authors have demonstrated that high alveolar bone density might be a risk factor for MRONJ (Takaishi, Ikeo, Nakajima, Miki, & Fujita, 2010). By measuring Computed Tomography (CT) values of cancellous bone, other authors verified that these imaging measurement could be considered as a potential method for detecting the early stages of MRONJ (Hamada, Matsuo, Koizumi, Satomi, & Chikazu, 2014). A recent study verified that CT values for cancellous bone at both affected and unaffected areas of MRONJ patients were significantly higher than those of the healthy group (Taniguchi et al., 2016). In animal models, Microcomputed Tomography (MicroCT) has been used for evaluating bony changes associated with MRONJ (Bi et al., 2010; Howie et al., 2015; Kim, Tatad, Landayan, Kim, & Kim, 2015).

MicroCT is the best imaging technique for evaluating bone morphology and microarchitecture in mice and other small animal models. The MicroCT devices achieve an isotropic voxel size of as low as few micrometers, which is sufficient for investigating structures such as mouse trabeculae that have widths of approximately 30–50 μm (Bouxsein et al., 2010).

Several previous animal models have utilized tooth extraction and high-dose bisphosphonates to reproduce clinical and imaging findings of MRONJ in humans. Nevertheless, the pathophysiology of the disease remains uncertain (Abtahi, Agholme, Sandberg, & Aspenberg, 2012; Ali-Erdem et al., 2011; Allen & Burr, 2008; Bi et al., 2010; Biasotto et al., 2010; Hokugo et al., 2010; Howie et al., 2015; Huja et al., 2011; Kobayashi et al., 2010; Marino et al., 2012; Perilli et al., 2010; Senel et al., 2010; Zandi et al., 2016). These aforementioned studies have not yet verified the time at which bony changes appear, as well as whether imaging findings precede clinical changes, such as bone exposure. We hypothesized that microtomographic bony changes may occur before dental extraction in rats receiving BP therapy, and that these bony changes can be found not only at the side of the local trauma, but also at the contralateral side, which had received the same pharmacological treatment but no tooth extraction. Therefore, the aim of this study was to evaluate early bony changes in an animal model of MRONJ at the side of the local trauma and at the contralateral side, comparing with a control group.

2. Materials and methods

2.1. Ethics statement

The study protocol was approved by the Ethics Committee in Animal Use of the University of Brasilia, Brazil (UnBDOC 5772/2015). The experimental procedures followed the ARRIVE guidelines, the National Institutes of Health guide for the care and use of Laboratory animals (NIH), as well as Brazilian Society of Laboratory Animal Science (COBEA).

2.2. Study design

2.2.1. Animals

The initial sample comprised forty female rats (*Rattus norvegicus*). The animals were randomly selected and housed in polypropylene cages (4 per cage) with average temperatures of 23° ± 2 °C and 12 h light/dark cycle. Water and food were provided *ad libitum*. After 2 weeks of acclimatization, the rats were randomly divided into two groups: Zoledronic Acid (ZA) and control groups. They were anesthetized and sedated (ketamine 10%, 90 mg/kg; xylazine 2%, 15 mg/kg) when necessary to perform the experiments.

2.2.2. Medication

Animals from ZA group received Zometa® 4 mg/5 ml (Novartis, Pharma, Basel, Switzerland), 66 μg/kg. Control animals received a saline solution in similar volume dosage. The rats were weighted before every experimental phase to properly dose the administered drugs, as well as for controlling weight gain or loss during the study. The medication was administered by intraperitoneal injection thrice a week, for three weeks according to Zandi et al. (2016). This protocol was previously tested in a pilot study. At the time of zoledronate administration, the mean age of the rats was 8 weeks and the mean weight was 250 g in accordance with previous studies (Bi et al., 2010; Ponte et al., 2016).

2.2.3. Dental extraction

Dental extractions were performed in animals of both groups (ZA and control) three weeks after beginning the bisphosphonate therapy. Animals were previously anesthesized with ketamine 10%, 90 mg/kg and xylazine 2%, 15 mg/kg. The lower left first molars were extracted by the same operator in both groups with a dental explorer. After the extraction, the animals were carefully assisted and analgesics were administered twice a day, for two days (tramadol, 60 mg/kg/day). The animals were weighed twice a week during the following six weeks. Behavior, ability to feed and presence of external injuries in the oral region were observed.

2.2.4. Visual examination

Clinical analyses were performed by a single examiner in a lighted room to visually determine the presence of bone exposure, abscesses or fistulae at the three phases of the experiment (T0, T1 and T2). The visual assessment also aimed to verify the onset of MRONJ after extraction.

2.2.5. Microtomography assessment

MicroCT scans were performed by using an *in vivo* SkyScan 1076 device (Bruker, Kontich, Belgium). The technical parameters were 18 μm pixel size, 100 kV, 100 μA, Al 1.0 mm filter, rotation step of 0.44, frame averaging of 5 and 37 min of average scanning time. The three-dimensional images reconstructions were obtained with ring artifact, smoothing and beam-hardening correction set at 14th position, one level and 55%, respectively (NRecon software with GPU acceleration, 1.6.9 version, Bruker, Kontich, Belgium). In total, the study comprised three different experimental phases of MicroCT evaluation.

T0: The baseline microCT examination occurred in experimental day 0, before medication and dental extractions, to analyze baseline qualitative and quantitative bony changes.

T1: The second microCT scanning was performed after three weeks of medication to investigate bony changes associated with the zoledronic acid intake, prior to the dental extractions.

T2: The last microCT images were acquired six weeks after the