Long-term therapy with intravenous zoledronate increases the number of nonattached osteoclasts

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ABSTRACT

The purpose of this study was to investigate the influence of long-term therapy with intravenous zoledronate (ZA) on the healing of extraction sockets in rats. Forty rats, divided into groups C (Control) and Z (Zoledronate), received intravenous injections of either saline solution or ZA for 24 weeks. Their right maxillary incisor was extracted. Euthanasia was performed at 7 or 28 days postoperative. Histomorphometric (Newly Formed Bone Area) and immunohistochemical (RANKL, OPG and TRAP) analyses were performed. Data were statistically analyzed (ANOVA, Tukey’s test and Kruskal-Wallis, Dunn’s Multiple Comparison test). Groups C and Z showed similar new bone area, RANKL and OPG immunolabeling. The number of TRAP-positive multinucleated cells was significantly higher in Group Z than in Group C at 28 days. A significantly higher proportion of nonattached osteoclasts were seen in Group Z than in Group C at both periods of analysis. Long-term therapy with intravenous ZA stimulated nonattached osteoclast formation in extraction sockets in rats, thus decreasing local bone resorption. However, it did not influence bone formation by osteoblasts.

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1. Introduction

Bisphosphonates (BPs) are antiresorptive drugs that are being used clinically to treat bone-related diseases, such as osteoporosis or cancer with bone metastasis (Williams et al., 2014). Users of these drugs are known to be at higher risk of developing osteonecrosis of the jaw (ONJ), which was first described by Marx (2003).

A systematic review and international consensus was published by a ‘task force’ sponsored by fourteen societies, including ASBMR, AAOMS, Canadian Association of Oral and Maxillofacial Surgeons, European Calcified Tissue Society, and Japanese Society for Bone and Mineral Research, among others. This task force defines ONJ as: 1) exposed bone in the maxillofacial region that does not heal within 8 weeks after identification by a health care provider; 2) exposure to an antiresorptive agent; and 3) no history of radiation therapy to the craniofacial region (Khan et al., 2015).

The incidence of ONJ is highest in the oncology patient population (1–15%), where high doses of BPs are used at frequent intervals (Khan et al., 2015). In the osteoporosis patient population, ONJ development still remains controversial (Williams et al., 2014). The vast majority of case series have described ONJ occurring at sites that have had oral surgery, particularly at extraction sites (Barash et al., 2011; Ferlito et al., 2011).

The pathophysiology of ONJ is not well understood. The development of ONJ appears to be multifactorial and related to the dose and duration of exposure to the antiresorptive agent, pre-existing...
oral disease profile, intervention with dentoalveolar oral surgery, and genetic polymorphisms (Khan et al., 2015).

Osteoclasts, which are thought to be the key cells involved in the effects of BPs on ONJ, have two distinct functions: bone resorption and maintenance of bone remodeling balance (Kim et al., 2016). Osteoprotegerin (OPG), receptor activator of nuclear factor-κB (RANK), and receptor activator of nuclear factor-κB ligand (RANKL) proteins are mediators of several cell processes, including bone metabolism. In bone metabolism, osteoblasts modulate osteoclast formation and bone resorption by producing OPG and RANKL (Manrique et al., 2015). OPG (Shahbouh et al., 1999; Suda et al., 1999; Manrique et al., 2015) is able to bind to RANKL preventing it from binding to RANK. By binding to RANKL, OPG inhibits osteoclast maturation, thus inhibiting osteoclastogenesis as well as lymphocyte development. Any imbalance in the communication between OPG, RANK, and RANKL proteins can lead to alterations in the quality of the formed mineralized tissue (Shahbouh et al., 1999; Suda et al., 1999; Manrique et al., 2015). Some studies have demonstrated that BPs inhibit the expression of RANKL, increase levels of OPG, and reduce osteoclast differentiation (Nakagawa et al., 2015; Mackie et al., 2001; Viersek et al., 2002). In contrast, Cardemil et al. (2015) observed similar expression of RANKL and OPG in alveolar bone samples from patients with and without ONJ after BP treatment.

Studies (Bi et al., 2010; Hokugo et al., 2010; Aghaloo et al., 2011; Aguirre et al., 2012; Williams et al., 2014) have unequivocally shown osteoclasts (Baron et al., 2011; Williams et al., 2014) to be the key mediators in causing ONJ. Nonetheless, direct evidence of involvement and the extent to which osteoclasts contribute to ONJ pathogenesis remains elusive. It remains unclear whether ONJ development by BPs is associated with their inhibitory effects on bone-resorptive functions of osteoclasts or with the aberrant behavior of osteoclasts (e.g. secretion of cytokines to the surrounding environment) after BP uptake (Williams et al., 2014). Current literature remains inconclusive with respect to the presence, numbers, and location of mature osteoclasts (Reid and Cornish, 2011; Williams et al., 2014). Cytotoxic effects of BPs on cells other than osteoclasts (e.g. endothelial cells or oral mucosa cells) have further complicated the roles of osteoclasts in the pathophysiologic characteristics of ONJ (Williams et al., 2014).

Several mechanisms have been proposed in an attempt to explain the pathophysiology of BP-related ONJ (BRONJ) (Diniz-Freitas et al., 2012; Silva et al., 2015), but we are far from understanding its pathogenesis. The pathophysiology of ONJ needs to be more clearly delineated using well-characterized animal models that lend themselves to better understanding the human condition (Khan et al., 2015). The purpose of this study was to histomorphometrically and immunohistochemically analyze the influence of long-term therapy with IV zoledronate (ZA) on the healing of healthy extraction sockets in rats.

2. Material and methods

2.1. Animals and study design

The experimental protocol was approved by the Univ. Estadual Paulista – UNESP, Dental School of Araçatuba Institutional Animal Care and Use Committee (# 2007-001204), SP, Brazil, and is in accordance with the EU Directive 2010/63/EU. Forty healthy male rats (Rattus norvegicus albinus, Wistar), 3–4 months old, weighing 350–400 g (UNESP, Dental School of Araçatuba, Animal Care Unit) were randomly divided into two groups: Z (zoledronate) and C (control). Group Z received IV injections of ZA (Zomethe™ 4 mg, Novartis Pharmaceuticals Corporation, East Hanover, NJ, USA; 76 μg/kg of body weight diluted in sterile 0.9% sodium chloride) once every 4 weeks over the course of 24 weeks. This dose was calculated by the pharmacology researcher (CA) to be equivalent to that used for oncology treatments. Under identical conditions, group C received IV injections of saline solution (0.15 M) containing no ZA. The solutions were administered in the jugular vein.

Under general anesthesia, tooth (healthy) extractions were performed 24 h after the last IV injection of either solution. Each group was divided into two sub-groups for euthanasia at 7 or 28 days postoperative (n = 10). All animals were euthanized with an overdose of pentobarbital (Thiopeptax, Cristália Produtos Químicos e Farmacêuticos Ltda., São Paulo, Brazil).

2.2. Tooth extractions

Following the six monthly injections (24 h after the last injection of either saline or ZA), the rats were anesthetized by an intramuscular injection of xylazine (6 mg/kg body weight) and ketamine (70 mg/kg body weight) and the healthy right maxillary incisor was extracted. The dental sockets were sutured.

2.3. Tissue processing

Animals were euthanized at either 7 or 28 days postoperative. The area of the extraction socket and the surrounding tissues were removed en bloc. The blocks obtained were fixed in 10% neutral formalin, rinsed with water, and then decalcified in 10% ethylenediaminetetraacetic acid (EDTA) solution. Following decalcification, they were processed and embedded in paraffin. Serial sections 6 μm thick were cut in a longitudinal direction and stained either with hematoxylin and eosin (H&E) for histomorphometric analyses, or subjected to the indirect immunoperoxidase detection method.

For the immunohistochemical reactions, the histologic sections were deparaffinized and rehydrated through a graded series of ethanol. For antigen retrieval, the slides were incubated in a buffer solution (Diva Decloaker®, Biocare Medical, CA, USA) in a pressurized chamber (Decloaking Chamber™, Biocare Medical, CA, USA) at 95 °C for 10 min. At the end of each step of the immunohistochemical reaction, the histological slides were washed with phosphate buffer solution (0.1 M, pH 7.4). The slides were immersed in 3% hydrogen peroxide for 1 h and 1% albumin bovine serum for 12 h to block the endogenous peroxidase and nonspecific sites, respectively. The slides containing samples of each of the experimental groups were divided into three batches and each batch was incubated with one of the following primary antibodies: goat anti-rat RANKL (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-rat OPG (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or goat anti-trap TRAP (1:180; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The sections were incubated with a biotinylated secondary antibody for 2 h and subsequently treated with a streptavidin-horseradish peroxidase conjugate for 1 h (Universal Dako Labeled HRP Streptavidin-Biotin Kit®, Dako Laboratories, CA, USA). The reaction was developed using the chromogen 3,3'-diaminobenzidine (DAB chromogen Kit®, Dako Laboratories, CA, USA) and counterstained with Harris hematoxylin. All samples were accompanied with a negative control.

2.4. Image analysis procedure

Five histological sections, representing the center of the original extraction socket, were selected for histological/histometric (2 sections) and immunohistochemical (3 sections) analyses. They were performed by examiners, calibrated and masked with respect to the treatment rendered, using image-analysis software (Axiovision 4.8.2, Carl Zeiss MicroImaging GmbH, Jena, Germany).
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